Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 103 FEBRUARY 1960 No. 2

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Mineralization of Dental Calculus.* (25479)

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Most previous studies on human dental calculus have dealt with gross morphology, chemical composition, mechanisms of deposition, and pathological effects upon adjacent tissues. The microscopic structure of calculus has been investigated almost exclusively in demineralized material(1). In recent studies of structural characteristics of calculus as revealed in ground sections, we observed that various configurations recur consistently in all specimens. An impression has been gained that calculus may be organized according to a specific pattern, in a sense resembling calcified tissues. It was quite obvious that through use of experimental technics information could be obtained which bears both upon formation and structural organization of calculus and upon deposition of mineral in organic matrices in general.

Material and methods. Sandblasted polyester strips (Mylar, DuPont) were fastened to the lingual surfaces of 73 lower incisors in 16 subjects who were known to form calculus rapidly(2,3). At various intervals, from 1 day to 4 weeks, the strips, with the deposit which had formed on them, were removed and processed for either optical or electron microscopy. Material to be examined optically was fixed in 10% neutral formalin, and embedded in celloidin. Serial sections (12μ) were stained with hematoxylin and eosin, alizarin red S, and by the Gram, von Kossa, and periodic acid Schiff (PAS) methods. For electron

* Supported in part by U.S.P.H.S. grant.

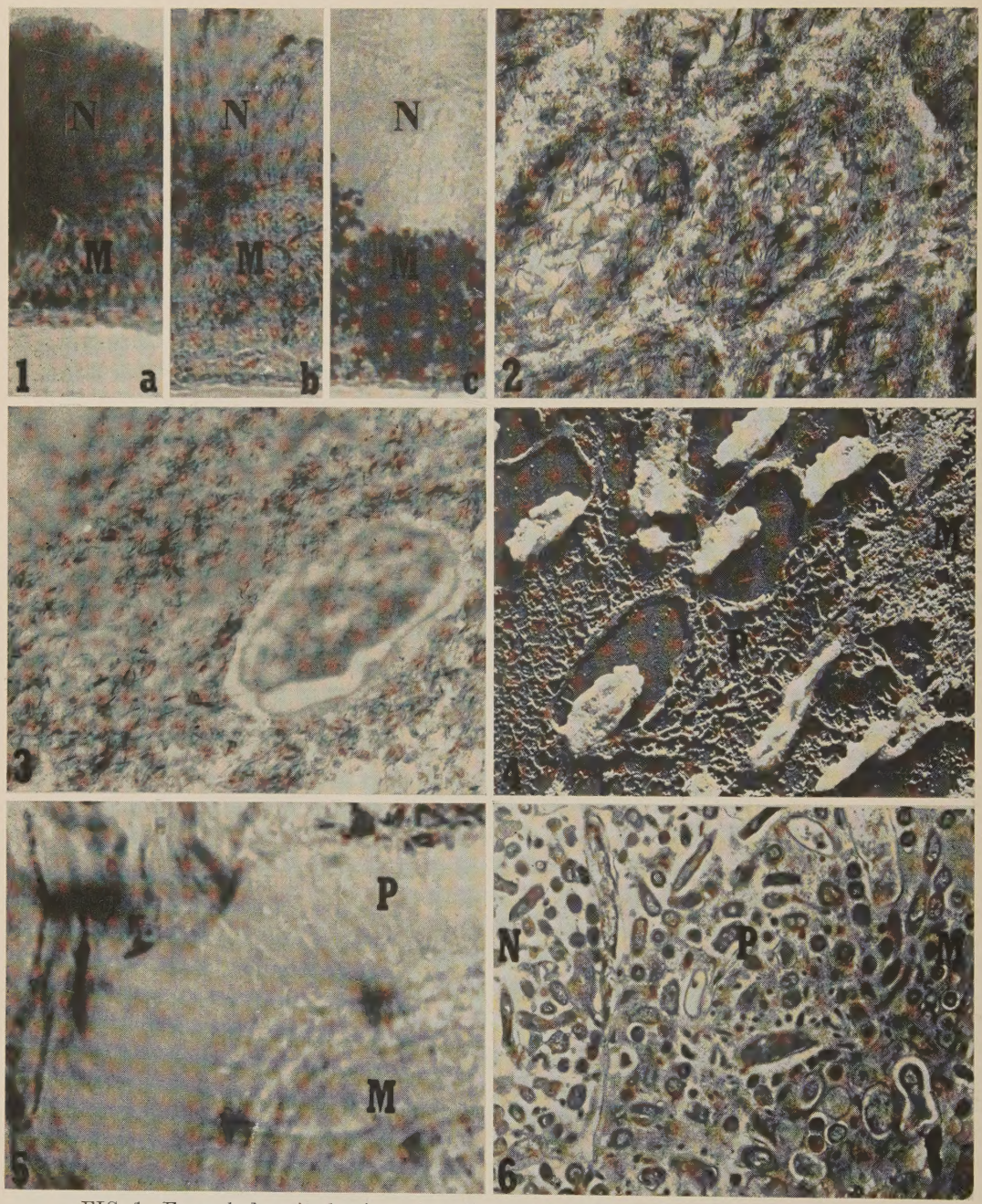

FIG. 1. Four-wk deposit showing mineralized region (M) and non-mineralized region (N) stained by H & E (a), Gram (b) and von Kossa (c) methods. Polyester strip is at bottom. Photomic., 510×.

FIG. 2. Needle-like crystals within and between microorganisms in a region of advanced mineralization. Electron mic., 25,000×.

FIG. 3. Crystals only between and on surfaces of microorganisms in a region of beginning mineralization (M). Electron mic., 25,000×.

FIG. 4. Organic intermicrobial material (P) immediately external to zone of beginning mineralization (M). Electron mic., 18,500×.

FIG. 5. The same 2 regions as in preceding figure. Photomic., 1790×.

FIG. 6. Differences in density in intermicrobial material in regions of recently formed (N), solidified (P) and mineralized (M) deposit. Electron mic., 4000×.

microscopy the specimens were fixed in an osmium tetroxide-potassium dichromate mixture and embedded in a mixture of methyl and butyl methacrylates. Sections were cut serially with a Porter-Blum microtome equipped with glass and diamond knives.

Results. The first signs of mineralization were observed in 3-day-old specimens. Areas adjacent to the polyester strips appeared clearly black with von Kossa stain, red with alizarin red S, were PAS negative and took H and E only faintly. Only a few microorganisms were brought out by the Gram stain. At 4 weeks the mineralized regions were considerably larger and more numerous, but retained the same staining properties (Fig. 1 a-c, area M). Under the electron microscope, the outlines of microorganisms could actually be discerned in these masses. Large numbers of needle-like crystals were seen throughout, both between and inside the microorganisms (Fig. 2). These crystals appeared as fine needles and gave electron diffraction patterns typical of apatite. Toward the outer border of the mineralized areas, where calcification was less advanced, apatite crystals were found on the surfaces of and between the microorganisms, but not within them (Fig. 3). When the plastic embedding material was removed from such sections and the preparations were shadowed, a fine fibrillar matrix was evident throughout the interbacterial region. An even more copious amount of this material was seen in the immediately surrounding areas, where mineral deposition had not yet begun (Fig. 4). Examined optically, the latter zone did not take alizarin red S or von Kossa stain, stained light blue with H and E, and was slightly PAS positive (Fig. 5 P). Still farther from the mineralized zone, the most recently deposited material was made up primarily of Gram positive microorganisms (Fig. 1b, area N); it stained deep blue with H and E, and was strongly PAS positive. Under the electron microscope the most striking feature of this area was the relative lack of density and definition of the interstitial substance (Fig. 6).

Discussion. From our observations it might be deduced that the sequential process of mineralization in calculus is the same as in calcified biological tissues. Thus, the microorganisms and intermicrobial material serve as the organic "matrix" which subsequently becomes impregnated with mineral crystals. The apparent increase in quantity and density of the intermicrobial substance shortly before inception of mineralization seems to be a counterpart of the solidification that occurs in the dentinal matrix during the same developmental period(4). The progressive changes in staining properties also indicate that basic alterations occur in composition of the initially deposited matrix, and the positive PAS reactions suggest that, as in tooth and bone, polysaccharide plays a role in the mineralization process. The pattern in which the apatite crystals are laid down is undoubtedly unique for calculus but it is of interest that the microorganisms themselves ultimately become calcified.

The most important aspect of this study seems to us to be the demonstration that calculus can be made to serve as a useful test object in basic studies of mineralization. Its ready accessibility and rapidity of its development present promising experimental possibilities. At the same time, extension of the work will also provide further information related directly to the composition and microstructure of calculus itself. One of the most challenging problems lies in identification of the organic intermicrobial substance and the tracing of its source.

Summary. Experimental studies have been made of mineralization in developing human dental calculus, formed *in vivo* on polyester strips attached to the teeth. The findings suggest a close parallelism between calcification process in this exogenous deposit and in calcified tissues. The microorganisms and intermicrobial substance first laid down serve as the organic matrix, which undergoes a series of chemical and morphological changes prior to mineralization. Calcification within this matrix follows a definite pattern, crystals first being laid down between and on surfaces of the bacteria, and later inside them.

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Received November 25, 1959. P.S.E.B.M., 1960, v103.

Prevention of Levarterenol Necrosis in Rabbits by Use of Levarterenol-Phentolamine Mixtures.* (25480)

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Levarterenol (Levophed), administered by slow intravenous drip, is the vasopressor drug most frequently employed in treatment of shock. Accidental extravasation may produce such intense local vasoconstriction that ischemic necrosis of the skin may ensue. Vasoconstriction can be reversed by prompt injection of the anti-adrenergic drug phentolamine (Regitine) into the area of extravasation (1,2). The method is not effective when extravasation has been overlooked for several hours and irreversible tissue damage has occurred. Neglect of an extravasation might not be serious if phentolamine could be given intravenously with levarterenol. Preliminary clinical studies indicate that levarterenol-phentolamine mixtures may be used effectively(3). The purpose of our study is to determine the least amount of phentolamine that will inhibit the local vasoconstrictor action of doses of levarterenol usually employed in treatment of shock.

Methods. The method described by McGinn and Schluger(4) was used for producing sloughs on rabbit abdomen. Solutions were prepared in 1000 cc of 5% glucose in water. They were administered through 22 gauge 1½ inch needles inserted subcutaneously at center of shaved abdomen and directed caudally. The drip was adjusted so that 250 cc were infiltrated during 2 hour period. The needles were then removed. Experiments were carried out on 36 adult rabbits, divided into 6 groups of 6 rabbits each. Group A served as control receiving only levarterenol.† Of this group, 2 were injected with 8 mg/l levarterenol, 2 with 16 mg/l, and 2 with 32 mg/l. Each of the other 5 groups was similarly subdivided. However, the levarterenol solutions for these animals contained increasing amounts of phentolamine‡: Group B, 0.5 mg; Group C, 1 mg; Group D, 2.5 mg; Group E, 5 mg and Group F, 10 mg.

Results. Group A: Subcutaneous infiltration of levarterenol solutions without phentolamine produced a well-demarcated swelling. After 24 hours, there was a mottled, warm area of induration. After 72 hours, necrosis with scaling and crusting occurred. Size of slough varied directly with concentration of levarterenol. The smallest measured 3 x 4 cm and the largest 12 x 20 cm.

Group B: The 2 rabbits that received the mixture with 8 mg levarterenol developed no necrosis. The 2 injected with 16 mg and the 2 with 32 mg developed areas of necrosis approximately the same size as those in corresponding animals in Group A.

Group C: No necrosis was observed in 2 rabbits that received the mixture with 8 mg levarterenol or in one with 16 mg. A 1 x 1 cm slough developed in the second rabbit of latter group. Necrosis appeared in both rabbits given the mixture with 32 mg levarterenol. One measured 2 x 2 cm and the other 0.5 x 2 cm.

Groups D. E. F: Necrosis did not develop in any of the animals.

Discussion. Experiments have confirmed

* We wish to thank Dr. William Antopol and Dr. Arthur M. Fishberg for valuable assistance.

† Levarterenol (Levophed) was provided by Winthrop Labs., N.Y., N.Y.

‡ Phentolamine (Regitine) was furnished by Ciba Pharm. Products, Summit, N. J.

OUTCOME OF SUBCUTANEOUS INFILTRATION OF LEVOPHED-REGITINE SOLUTIONS IN 36 RABBITS

LEVOPHED, mg./L.	GROUP: A	B	C	D	E	F
32	++	++	++	oo	oo	oo
16	++	++	+o	oo	oo	oo
8	++	oo	oo	oo	oo	oo

0 0.5 1.0 2.5 5.0 10.0

REGITINE, mg./L.

+ NECROSIS o NO NECROSIS

FIG. 1. Effect of varying concentrations of phentolamine in prevention of levarterenol necrosis.

the previously reported observations that ischemic necrosis uniformly follows slow subcutaneous infiltration of 250 cc of levarterenol solution into the rabbit abdomen. McGinn and Schluger(4) used a solution equivalent to 16 mg of levarterenol/liter. Similar changes were produced in our study with solutions containing 8, 16, and 32 mg levarterenol. Size of sloughs varied directly with concentration of levarterenol.

Phentolamine given simultaneously with levarterenol has a potent inhibitory effect on cutaneous vasoconstriction of the latter (Fig. 1). As little as 0.5 mg counteracted the effect of 8 mg of levarterenol. A concentration of 1 mg phentolamine diminished the vasoconstriction produced by 16 and 32 mg levarterenol judging by the small size of resultant slough. The lowest phentolamine dose to abolish the ischemic effect of all 3 strengths of levarterenol was 2.5 mg. The same beneficial response was obtained with 5 and 10 mg phentolamine.

These experiments were carried out to determine smallest amount of phentolamine that will abolish the local vasoconstrictor action of doses of levarterenol usually administered to patients in shock. If results in normal rabbits can be extrapolated to patients in shock, then mixtures containing 2.5 mg phentolamine might be free from the ischemic hazard. However, circulation in extremities of patients in shock is not comparable to that in the skin of abdomen of normal rabbits. Intense vasoconstriction commonly associated with shock may increase the requirement for phentolamine.

Summary. 1. Subcutaneous infiltration of 250 cc of solutions containing 8, 16, or 32 mg levarterenol/liter for 2 hours regularly produced slough of skin on abdomen of rabbits. 2. All levarterenol-phentolamine mixtures that contained 2.5, 5, and 10 mg phentolamine did not produce slough. Those containing 0.5 and 1 mg phentolamine prevented slough only for mixtures with 8 mg levarterenol. Sloughs were reduced with mixtures containing 16 and 32 mg levarterenol and 1 mg phentolamine.

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Received October 2, 1959. P.S.E.B.M., 1960, v103.

Serotonin Studies on Mouse Tissues.* (25481)

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Anaphylaxis in the mouse apparently differs from that observed for other species with respect to difficulty of sensitization and shock organs and mediators concerned. Since histamine is believed to be of relatively little importance in this reaction(1), attention has

* Supported by grant from N.I.H., U.S.P.H.S.

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TABLE I. Serotonin Content of Mouse Tissues.

Tissue	Serotonin values		
	Normal	Tumor*	Tumor-anaphylactic shock
Kidney	1.3 ± .1 (4)†	39.8 ± 13.7 (4)	9.9 ± 12.6 (5)
Lungs	2.7 ± .8 (2)	27.1 ± 12.2 (3)	3.5 ± 1.3 (6)
Small intestine	3.1 ± .3 (11)	12.9 ± 6.4 (6)	1.1 ± .9 (5)
Liver	0 ±0 (8)	81.6 ± 33.2 (10)	20.9 ± 5.6 (7)
Spleen	5.9 ±2.0 (3)	213 ± 40.4 (5)	175 ± 37.3 (7)
Tumor		169 ± 36.2 (6)	192 ± 41.8 (7)
Ascitic fluid		44.1 ± 80.1 (2)	

* Bearing actively growing, palpable mastocytoma.
† γ/g ± S.D. No. in parentheses = No. of determinations.

been focused on serotonin(2-5). The latter is of particular interest in this species because of abundance and widespread presence of large numbers of mast cells in the loose connective tissue of skin, snout, paws, etc. and along lymphatic channels, small blood vessels and nerves of forestomach, tongue, lungs, mesentery, intestines, etc.(6). The mast cell is probably responsible for secretion and liberation of serotonin(7). There is no evidence (4) that serotonin is released from tissues other than platelets in the anaphylactic reaction. A study of anaphylaxis in presence of a transplantable mouse mastocytoma, consisting essentially of mast cells and potentially capable of secreting large quantities of serotonin, indicated that neither presence of tumor nor its secretory products had any effects on the reaction(8). Serotonin content of tissues of tumor bearing mice was determined and the results compared to those obtained from normal mice. Alteration in serotonin content was subsequently determined in these mice following anaphylactic reaction.

Methods. The mice used were of LAF_1 strain treated as described previously(8). For purposes of serotonin determinations, the animals were divided into 3 groups. Group I consisted of control animals. Groups II and III consisted of mice bearing transplantable mast cell tumors. The animals in Group III were those which were sensitized and in which anaphylactic shock was elicited. Tissues were frozen immediately in a dry ice-cellosolve bath after animals were sacrificed or died in anaphylactic shock. The 2 kidneys, liver, small intestine and tumor from individual mice each provided sufficient tissue for serotonin analysis. It was necessary, however, to pool the lungs and spleens from several animals to measure tissue serotonin levels. Serotonin was assayed by the spectrophotofluorimetric method of Bogdanski *et al.*(9).

Results. Tissue serotonin levels of above 3 groups are presented in Table I. Tissue content of serotonin in mice bearing the transplantable mastocytomas was markedly increased, and most increased in liver and spleen. A high serotonin content of mast cell tumors was also found. Ascitic fluid from 2 mice with mastocytomas contained significant quantities of serotonin.

When tissue serotonin of tumor animals was compared to that observed in tumor mice subjected to anaphylactic shock, a significant decrease, ($P<.01$), in serotonin concentration was noted in kidneys, lungs, small intestine and liver. Serotonin content of small intestine and lung was reduced to values approaching those observed in control animals. Following anaphylaxis a decrease in serotonin content was not found in spleen or mast cell tumor.

Discussion. Although significant amounts of serotonin have been found in transplantable mastocytomas of mice(10), information concerning serotonin levels of various tissues of mice bearing this tumor has not been previously available. Our data suggest that a marked increase in serotonin content may be observed in tissues of such animals. The highest serotonin levels were found in spleen, where the amine is probably not being metabolized but is apparently being stored in platelets.

Previous studies showing a relatively high concentration in the lung have implicated serotonin in the anaphylactic reaction in the mouse(3). It is of interest that significant decreases in tissue serotonin content were ob-

served during anaphylaxis in the mast cell tumor mice. Kidneys, lungs, small intestine and liver are all thought to be capable of metabolizing free serotonin(11), and, in these organs, anaphylaxis was associated with a striking decrease in the elevated serotonin levels. A similar reduction was not noted in spleen or in mast cell tumors. Presumably in these tissues serotonin was either not released or, if released from cells, was not subsequently metabolized. Reduction in serotonin content of whole blood is accompanied by a fall in number of platelets during anaphylaxis in the rabbit(4). However, no evidence of release from tissue has been obtained(5). In tissues of mice bearing mastocytomas the decreases in serotonin concentration were striking.

The high serotonin content observed in tissues of mice with mast cell tumors suggest that such experimental animals might prove useful in the study of effects of serotonin in a situation where the amine is chronically present in large amounts. The mechanisms by which serotonin is bound to or released from tissues remain poorly understood. In view of the striking changes observed in tissue serotonin content, further studies utilizing organs of mice with mast cell tumors may help clarify these mechanisms.

Summary. Tissues of mice bearing mastocytomas are rich in serotonin. A significant decrease in serotonin content occurred in kidneys, lungs, small intestine and liver of these mice following anaphylactic shock.

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Received October 21, 1959. P.S.E.B.M., 1960, v103.

Absorption of Cholesterol-4-C^{14} Oleate* (25482)

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Studies(1,2) using blood cholesterol levels as an indicator of cholesterol absorption in chicks and rats have demonstrated that feeding of free cholesterol leads to a higher blood cholesterol level than feeding of the long chain fatty acid esters of cholesterol. Vahouny and Treadwell(3) examined lymph after feeding of different cholesterol esters and found that free cholsterol was absorbed more rapidly than the long chain fatty acid esters of cholesterol. They suggested that only free cholesterol was transferred from the lumen into the mucosa. Pihl(4) reported that fed cholesterol esters were extensively hydrolyzed in the intestine and were not absorbed to a greater extent than free cholesterol. Favarger and Metzger(5) fed D-cholesterol oleate and demonstrated hydrolysis of this labeled ester in the lumen of intestine. They also found that in the intestinal wall of such animals the labeled cholesterol was present principally in the free form. When free D-cholesterol was

* This investigation was aided by research grants from U.S.P.H.S. and Am. Heart Assn.

TABLE I. Cholesterol and Cholesterol-4-C^{14} of Lymph and Intestine after Feeding Cholesterol-4-C^{14} and Cholesterol-4-C^{14} Oleate.

Tissue*	Free	Free-C^{14}	Ester	Ester-C^{14}	Total	Total-C^{14}	Ester / Total	Ester-C^{14} / Total-C^{14}	Administered C^{14}-recovered
	mg						%		
				Fed cholesterol-4-C^{14} oleate					
Lymph	1.2 ±.3†	.3 ±.1	3.6 ±.5	1.9 ±.3	4.8 ±.4	2.2 ±.3	75.0 ±2.1	86.4 ±3.2	5.3 ±.6
Small intestine	14.1 ±.6	2.4 ±.3	2.6 ±.3	.8 ±.2	16.7 ±.5	3.2 ±.3	15.6 ±1.8	25.0 ±2.2	7.7 ±.6
				Fed cholesterol-4-C^{14}					
Lymph	1.9 ±.3	.6 ±.2	6.7 ±.4	4.1 ±.3	8.6 ±.5	4.7 ±.3	77.9 ±3.0	87.2 ±3.1	11.4 ±.7
Small intestine	14.4 ±.5	4.3 ±.3	2.6 ±.2	1.4 ±.2	17.0 ±.6	5.7 ±.4	15.3 ±1.2	24.6 ±2.1	13.8 ±.8

* Values are avg for 5 rats killed 6 hr after test meal. Cholesterol-4-C^{14} and cholesterol-4-C^{14} oleate administered/rat was 41.3 mg as free sterol (5000 cpm/mg).

† Stand. error of mean.

fed they were unable to demonstrate any esterified labeled cholesterol in intestinal lumen or wall. Evidence has been presented(6) that free cholesterol after transfer from the lumen is mixed with a pool of free cholesterol in the intestinal wall, then a major part is esterified and transferred to lymph. According to this concept, cholesterol esterification occurs at site of transfer of cholesterol from mucosa to lymph. The importance of this esterification step in cholesterol absorption has been questioned(4,5) since fed cholesterol esters are not absorbed to a greater extent than free cholesterol. However, if only free cholesterol can enter the intestinal wall, cholesterol esters must be hydrolyzed in the lumen prior to transfer of cholesterol into intestinal mucosa. If so, the esters might be absorbed at a slower rate than free cholesterol rather than the reverse.

Methods and materials. Rats with a thoracic duct fistula were prepared and given saline to drink(7). Twenty-four hours after operation each animal received, by gastric intubation without anesthesia, 3 ml of aqueous emulsion containing 50 mg albumin, 150 mg glucose, 292 mg oleic acid, 279 mg sodium taurocholate and either 41.3 mg cholesterol-4-C^{14} (0.5 μc) or 69.4 mg cholesterol-4-C^{14} (0.5 μc) equivalent to 41.3 mg cholesterol-4-C^{14}. Cholesterol-4-C^{14} oleate was synthesized according to the method of Page and Rudy (8) and had a m.p. of 44°C. Lymph was collected for 6 hours after administration of test meal and the animals were then sacrificed. The intestine was removed and washed with saline. The saline washings from intestine were pooled with the collected feces of the respective animal. Lipid extracts of intestine, lymph, and combined feces and intestinal contents were prepared according to procedures described earlier(6,7). Free and total cholesterol of each extract were determined colorimetrically by the method of Sperry and Webb(9). C^{14}-activity of free and esterified cholesterol fractions of all lipid extracts was assayed as previously described (6,7).

Results (Table I). Six hours after test meal containing cholesterol-4-C^{14} oleate, 5.3% or 2.2 mg of the fed cholesterol was present in lymph and 7.7% or 3.2 mg in the intestinal wall. Of the total C^{14}-cholesterol in lymph, 86.4% was esterified while only 25.0% of the total C^{14}-sterol was present as ester in the intestinal wall. The group fed free cholesterol-4-C^{14} had approximately twice the amount of C^{14}-cholesterol in both lymph (11.4% or 4.7 mg) and small intestine (13.8% or 5.7 mg) that was present in the group fed cholesterol-4-C^{14} oleate. The percentage of esterified cholestrol-4-C^{14} in lymph (87.2%) and small intestine (24.6%) of the cholesterol-4-C^{14} group, were the same as in the group fed the C^{14}-ester. There were no differences in chemical cholesterol levels of the intestine of the 2 groups. However, lymph of the cholesterol-4-C^{14} group had

TABLE II. Recovery of Fed Cholesterol-4-C^{14} and Cholesterol-4-C^{14} Oleate from Feces and Contents.

Free	Free-C^{14}	Ester	Ester-C^{14}	Total	Total-C^{14}	Administered C^{14}-recovered, %
mg						
Fed cholesterol-4-C^{14} oleate*						
10.7 ± 1.4	6.4 ± 1.6	23.5 ± 3.1	22.2 ± 2.6	34.2 ± 4.0	28.6 ± 3.4	64.3 ± 5.0
Fed cholesterol-4-C^{14}*						
29.4 ± 3.6	26.2 ± 4.0	2.0 ± .6	Tr	31.4 ± 3.1	26.2 ± 4.0	63.4 ± 6.0

* See Table I for amount of sterol fed.

larger amounts of both free and ester cholesterol than lymph of the C^{14}-ester group.

Examination of feces and contents following feeding of cholesterol-4-C^{14} oleate Table II) indicated that while extensive hydrolysis of the fed C^{14}-ester had occurred in the intestinal lumen, a large portion (78.0%) of the fed material was still present as unhydrolyzed ester. There was also considerably more *unlabeled* free cholesterol than *unlabeled* ester cholesterol. In the group fed free cholesterol-4-C^{14} virtually all cholesterol recovered from the lumen and feces, was present as free C^{14}-cholesterol. Also, there was very little unlabeled esterified cholesterol present in the contents. Total unlabeled sterol present in feces and contents of both groups was approximately the same. Total recovery of fed cholesterol-4-C^{14} oleate from feces and contents, lymph and intestine was 82.3%. Thus, 17.7% could not be recovered and may have undergone intestinal transformation as reported earlier(6). The balance data on the cholesterol-4-C^{14} group indicate that 11.4% of the fed dose was not recovered.

Discussion. Results of our study have confirmed, and extended earlier observations(2,3) and provided more definitive data regarding the first step in absorption of cholesterol. There is extensive hydrolysis of fed cholesterol esters in the lumen of intestine, but virtually no esterification of fed free cholesterol occurs in this area of the intestine. These findings agree with those of Pihl(4) and Favarger and Metzger(5). If fed cholesterol-4-C^{14} oleate were absorbed preferentially without being hydrolyzed in the lumen, it would be expected that most of the C^{14}-activity in the intestinal wall would reside in the esterified fraction. Also, there would be very little C^{14}-activity in the free fraction of lymph. The data indicate that following feeding of either cholesterol-4-C^{14} oleate or cholesterol-4-C^{14} most of the C^{14}-cholesterol was transferred from the lumen into the intestinal wall as free sterol. Regardless of whether the free or the ester were fed, amount of C^{14}-ester esterified in intestinal wall was the same. The data on lymph show that most of the fed C^{14}-free or C^{14}-ester was present in lymph as C^{14}-ester. This would suggest, in line with our previous findings(6), that the esterified fraction of lymph originates from the free cholesterol pool of the mucosa and that esterification occurs in transfer of this sterol from intestinal wall to lymph. The greater amounts of cholesterol-4-C^{14} in lymph and intestine of the animals fed free cholesterol-4-C^{14} than when cholesterol-4-C^{14} oleate was fed, must be due to rate-limiting hydrolytic reaction occurring in the lumen of intestine prior to entrance of cholesterol into the intestinal wall.

Summary. Cholesterol-4-C^{14} oleate and cholesterol-4-C^{14} were fed in a test meal to lymph fistula rats. The data indicate that free cholesterol was absorbed to a greater extent than esterified cholesterol. This study also provides evidence to support the concept that preliminary hydrolysis is obligatory for absorption of cholesterol esters and that only free cholesterol can enter the intestinal wall. The significance of these findings is discussed.

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Received October 29, 1959. P.S.E.B.M., 1960, v103.

Tolerance of F_1 Hybrid Skin Homografts in the Parent Strain Induced by Parabiosis.* (25483)

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Immunological tolerance allowing homotransplantation of tissues and organs has been achieved by exposing animals to donor strain antigens prior to delivery or during immediate neonatal period(1). However, our recent reports indicate that weanling Z mice, injected with viable spleen cells taken from (Z x Ce) F_1 hybrid individuals, became tolerant and accepted skin grafts from (Z x Ce)F_1 donors (2). Similarly, older female mice of A and C_{57}Bl (Subline 1) strains were made tolerant of male skin isografts either by intravenous injection of male spleen cells or by establishing a parabiotic union of adult females with isologous males(3). Further, using the technic of parabiosis, it has also been demonstrated that tolerance of homologous tissue transplants, induced during neonatal period, can be transferred to normal adult mice of the same strain. In the latter experiment, the previously non-treated parabiont lost its ability to reject homologous tissue and, like its partner, accepted the homologous skin graft (4). Therefore, it seems that under certain well-defined conditions, induction of immunological tolerance in mice may not be limited to antigen exposure during embryonal life or immediately after birth. Our incidental observations indicate that, contrary to expectation, parabiosis performed between certain F_1 hybrid mice and individuals of either parent strain were successful in most instances and resulted in induction of tolerance in the parent parabiont whereby this animal lost its capacity to recognize and reject homologous skin grafts taken from hybrid donors of the corresponding F_1 cross. It is the purpose to document and further extend these observations.

Method. Highly inbred mice of the Z§, Ce, A, C_{57}Bl (Subline 1) and their reciprocal F_1 hybrids were used. In one group of experiments parabiosis was established between (Z x Ce)F_1 hybrids and Z animals, and between (Z x Ce)F_1 and Ce. In this group mice were from 40 to 70 days of age at time of parabiosis. In another group parabiosis was performed between (A x Z)F_1 and Z, and between (A x Z)F_1 and A mice. These animals were from 30 to 46 days old when placed in parabiosis. In a third group (Z x C_{57}Bl)F_1 and Z, and (Z x C_{57}Bl)F_1 and C_{57}Bl mice were similarly united in parabiosis. These animals were from 30 to 50 days old when parabiosis was established. In all instances parabiotic animals were of the same sex. Each preceding group had its own controls, consisting of parabiotic pairs of either parental strain, *i.e.*, Z to Z and Ce to Ce for the first group, Z to Z and A to A for the second, and C_{57}Bl to C_{57}Bl and Z to Z for the third group. Parabiosis was of the celomic type(5-6), consisting essentially of surgical union of animals through midlateral incisions comprising skin, muscle and peritoneal cavities. Parabiotic pairs were housed individually in plastic mouse cages with free access to Purina Fox Chow and tap water. Parabiosis was maintained from 20 to 45 days, after

* Aided by grants from the U.S.P.H.S. and Minn. Division, Am. Cancer Soc.

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§ Z is used to simplify designation of mice of the C_3H strain, originally obtained in 1956 from Dr. John J. Bittner's mouse colony.

TABLE I. Immunological Tolerance of Homologous F_1 Hybrid Skin Grafts Induced in Mice of Their Parent Strains by Way of "F_1 to Parent" Parabiosis.

Parabiosis groups		No. of pairs	Age when joined (days)	Time in parabiosis (days)	No. of pairs dead*	Successful F_1 hybrid skin graft on parent parabiont†
(Z × Ce) F_1	Z	28	45–70	35	2	26/26‡
Idem	Ce	12	40–50	20	2	10/10
Z	Z	28	53–72	35	4	0/24
Ce	Ce	14	34	20	4	0/10
(A × Z) F_1	Z	16	36–46	45	3	12/13
Idem	A	15	30–35	22	12	0/3
Z	Z	16	35–45	25	3	0/13
A	A	14	25	20	4	0/10
(Z × C_{57}Bl) F_1	Z	14	35–45	45	4	2/10
Idem	C_{57}Bl	12	30–35	25	4	0/8
Z	Z	12	44	20	2	0/10
C_{57}Bl	C_{57}Bl	12	50	20	2	0/10

* Death occurred prior to skin grafting. † Grafts were performed 8 days after disruption of parabiosis. ‡ No. of successful grafts/No. grafted.

which parabiosis was surgically discontinued under nembutal anesthesia, closing each peritoneal cavity with 5-0 catgut sutures, and both muscle and skin layers with metal clips. Eight days after disruption of parabiosis, the parent member of the pair received a full-thickness skin graft taken from hybrid donor of corresponding F_1 cross. In all instances donor and recipients were of the same sex and approximately the same age. The technic for skin graft was as previously described(1). In most instances, mice were kept under observation for at least 5 months following skin transplantation. In evaluating success or failure of graft, the direction of the hair growth was of primary importance, since the method involves a 180° rotation of donor skin prior to implantation on recipient. Further, successful grafts were regularly noted to increase in size with growth of host.

Results. The results obtained are summarized in Table I. Of 28 parabiotic pairs of (Z x Ce)F_1 with Z that were kept in parabiosis for 35 days, 2 died prior to skin grafting. All remaining 26 parent Z strain parabionts were tolerant and accepted the (Z x Ce) F_1 skin graft. Similar results were obtained in Ce animals held in parabiosis with (Z x Ce)F_1 hybrids, since in all instances the Ce parent parabionts were tolerant of this hybrid skin. On the other hand, none of the Z to Z or Ce to Ce parabiosis control animals accepted the F_1 hybrid graft.

In the group of (A x Z)F_1 joined in parabiosis with Z animals, 3 of 16 died prior to skin grafting. Of the remaining 13 Z parabionts, 12 were tolerant and accepted skin grafts from (A x Z)F_1 hybrid donors. Control Z animals which had been in parabiosis with Z animals regularly rejected the (A x Z)F_1 skin. In contra-distinction, in group in which parabiosis was established between (A x Z)F_1 hybrid and A parent strain mice, 12 of 15 pairs died during parabiotic union, and none of the 3 remaining parent A strain parabionts accepted the F_1 skin graft. As in other studies, the controls for this group always rejected the F_1 skin graft. Finally, in the group in which parabiosis was established between (Z x C_{57}Bl)F_1 and Z animals, of a total of 14 pairs prepared, 4 died during parabiosis. Of the remaining 10 pairs, only 2 of the Z parabionts were tolerant and accepted the (Z x C_{57}Bl)F_1 hybrid skin graft. In the group in which parabiosis was established between (Z x C_{57}Bl)F_1 and the C_{57}Bl parent, 4 out of 12 pairs died during parabiotic union, and none of remaining eight C_{57}Bl partners accepted subsequent F_1 hybrid skin graft. Here again, control Z animals that were in parabiosis with isologous Z mice, and C_{57}Bl animals united to animals of the same strain, all rejected skin grafts taken from (Z x C_{57}Bl)F_1 hybrid individuals.

Discussion. The results seem to demonstrate that by joining in parabiosis F_1 hybrid mice resulting from the cross between 2 inbred strains of mice with animals of either of

their parent strains, it is sometimes possible to obtain immunological tolerance of F_1 skin homotransplantation in the parent parabiont. In the second place, they confirm our previous observations indicating that by this method tolerance can sometimes be induced in adult individuals(3). This is clearly seen when parabiosis was established between (Z x Ce)F_1 and individuals of either of their parent strains, *i.e.*, Z or Ce. Similar results were obtained in Z animals joined in parabiosis with (A x Z)F_1 hybrids. However, when these hybrids were joined in parabiosis with mice of the A parent strain, death occurred in 80% of parabionts, and in those surviving parabiosis, the A parent parabiont rejected the F_1 hybrid skin homograft in every instance. Thus, a real difference between A and Z animals with respect to susceptibility to induction of tolerance by this manipulation seems apparent.

When (Z x C_{57}Bl)F_1 mice were placed in parabiosis with Z animals, 29% of the animals died, and of the surviving Z strain parabionts, only 20% became tolerant and accepted the corresponding F_1 hybrid skin graft. Further, parabiosis established between this same hybrid and C_{57}Bl parent strain also led to death in approximately 30% of parabionts, and the procedure failed to induce tolerance in any of the C_{57}Bl mice surviving parabiotic union. It seems clear that, whereas immunological tolerance permitting subsequent homotransplantation of skin can be achieved in certain strains of mice as a result of placing mice in parabiosis with a member of an F_1 combination, members of the other parent strain similarly treated, will not become tolerant. This result is particularly clear when the effects of parabiosis between (A x Z)F_1 and Z are compared with those obtained with (A x Z)F_1 and A.

It appears that strain differences exist with respect to capacity both to induce and accept tolerance by the method of parabiosis. Interpretation of these results is difficult. The hypothesis previously advanced to explain induction of tolerance in weanling Z mice by intravenous injection of spleen cells taken from (Z x Ce)F_1 hybrids(2) and tolerance of male skin in females of the same strain after parabiosis(3), is attractive. According to this concept, weak histocompatibility differences are postulated to exist between members of these combinations, and cells capable of being conditioned are assumed to be present in the reticuloendothelial or lymphoid tissues throughout life. When the histocompatibility differences are slight, parabiosis can be established and tolerance is eventually accomplished as cells from the hybrid enter the body of the parent parabiont. Strong histocompatibility differences between parabionts would be expected to preclude such a result, since the parabiotic union would fail primarily as a consequence of immunological interaction between the partners.

The results obtained in the second group of experiments in which Z animals were made tolerant of (A x Z)F_1 tissue, whereas A mice did not become tolerant when joined in parabiosis with the same F_1 hybrid, are more difficult to explain. Since these strains differ at the H_2 genetic locus, this hypothesis provides no explanation for susceptibility of Z animals to development of tolerance and resistance of the A strain animal to this phenomenon. However, a possible explanation for such a discrepancy is that genic interaction, such as that postulated by Fox(7) might have occurred by crossing these 2 strains, and there is lesser histocompatibility difference between Z and the (A x Z)F_1 than exists between A and the (A x Z)F_1. Such an occurrence might make it easier to establish parabiosis between Z and (A x Z)F_1 and also easier to develop tolerance once parabiosis has been established.

The mechanism responsible for induction of tolerance by parabiosis is not known. In this regard it seems worthy of considering the similarity of this situation to that demonstrated to exist by Egdahl and Hume in cross-circulated dogs(8). These authors found that, during period of cross circulation with donor, dogs would not reject homotransplanted kidney, even though they had been previously immunized, whereas termination of cross circulation resulted in almost immediate evidence of rejection of the kidney homotransplant. Kamrin's(9) observation that parabiosis in the rat may permit prolonged survival of renal tissue homotransplants is

also pertinent. Whatever the ultimate explanation for these observations, it is clear that in appropriate strain combinations a form of immunological tolerance can be achieved by parabiosis in mature animals. Although strains of mice used and the explanation of the phenomenon were somewhat different than that provided here, Rubin(10) using small number of mice has made similar observations.

Summary. 1) Tolerance of F_1 hybrid skin homografts in mice of their corresponding parent strains has been attempted by establishing "F_1 to parent" parabiosis. 2) Results were: a) Mice of either Z or Ce strain joined in parabiosis with (Z x Ce) F_1 hybrids became tolerant and accepted skin homografts taken from this hybrid. b) Tolerance of (A x Z)F_1 skin was also obtained in Z strain mice following parabiosis with (A x Z)F_1 hybrids. However, A mice joined to the same hybrids failed to develop tolerance and rejected the F_1 grafts in all instances. c) While 20% of Z mice became tolerant of (Z x C_{57} Bl)F_1 skin after being in parabiosis with this hybrid, none of C_{57}Bl parent parabionts were susceptible of the hybrid skin grafts.

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Received November 12, 1959. P.S.E.B.M., 1960, v103.

Delayed Repolarization in Smooth Muscles. (25484)

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A delay in repolarization following spike depolarization gives a plateau in heart muscle normally(1), in squid giant axons after injection of tetraethyl amine(2), and in mylinated nerve in presence of barium(3). The plateau in heart muscle can be varied in duration by a variety of treatments and appears to depend largely on a delayed rise in potassium conductance(4). The plateau in frog ventricular muscle is lengthened by barium(5). A plateau-type potential normally occurs in the ureter(6) but is unlike that in heart in being prolonged by quaternary ammonium compounds and shortened by epinephrine and low calcium(7). In other smooth muscles the spike normally shows equal rates of depolarization and repolarization(8,9,10).

Experimental. Action potentials were recorded from cat intestinal muscle by means of the sucrose gap electrode(11). When 0.1% barium chloride was added to the perfusing Krebs solution, 45 mv. spikes appeared which were soon transformed into plateaus (Figs. 1 and 2). The duration was increased from a spike of about 100 msec to depolarizations which persisted for as long as 70 seconds. The effect was reversible, as shown by washing out the barium (Fig. 1-c, d). The longer plateaus were usually characterized by oscillations, especially just prior to their termination (Fig. 1-b, Fig. 2-a, b, c).

Plateaus with trains of spikes on their crests were characteristically produced in cat longitudinal intestinal muscle by acetylcholine (10^{-6}) (Fig. 3-a, b). A higher concentration (10^{-5}) produced maintained depolarizations accompanied by spike activity (Fig. 3-c) as described for taenia coli(12). A critical con-

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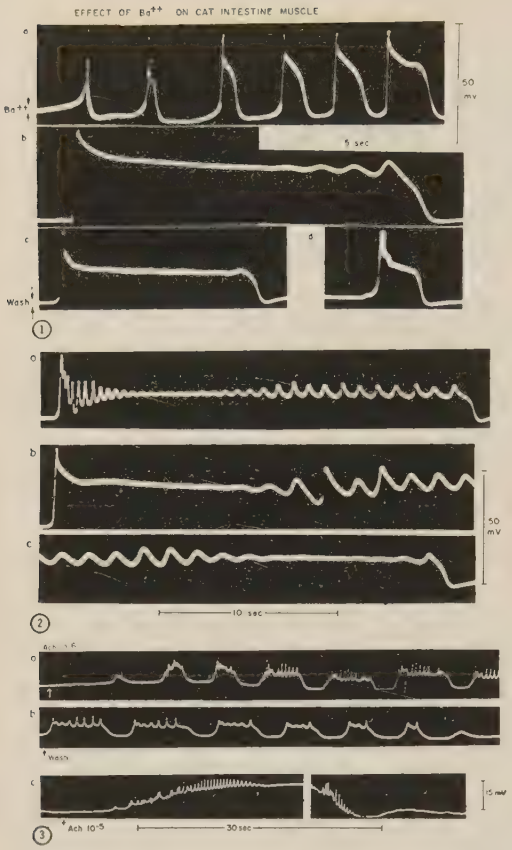

FIG. 1. Effect of 0.1% BaCl solution on cat intestine circular muscle. Sucrose gap technic. 37°C. a. Transition from normal to plateau type spikes during first 20 sec. exposure to barium. b. Typical plateau after 60 sec. c, d. Reversal of plateau to normal type spikes, 20 sec. and 50 sec. after washing out barium respectively.

FIG. 2. Examples of oscillations appearing on crests of barium (0.1%) induced plateaus. Cat intestine circular muscle. Sucrose gap technic. 37°C. a. Single plateau, with oscillations at beginning and end. b, c. Record of a single plateau during the first 25 sec. (b) and last 25 sec. (c). The central 15 sec. oscillation has been omitted.

FIG. 3. Effect of *acetylcholine* on cat intestine longitudinal muscle. Sucrose gap technic. 37°C. a. Development of plateaus topped by spikes during the first 50 sec. exposure to Ach 10^{-6}. b. Gradual reduction of plateaus on washing out Ach 10^{-6}. c. Maintained depolarization topped by spikes produced by Ach 10^{-5}. d. Repolarization on washing out acetylcholine by saline.

centration determines whether the response to acetylcholine is plateau-like or a maintained depolarization with multiple spikes. In taenia coli, barium depolarizes as does acetylcholine without producing plateau-type spikes. In muscularis mucosae of pig esophagus both acetylcholine and epinephrine cause prolonged depolarization and spikes, but *not* plateaus.

Evidence is insufficient to decide whether the plateaus result from maintained Na conductance, from delayed K conductance or from a combination of both factors. The oscillations occurring on the crests of the plateau may indicate a delicate balance of Na and K permeabilities.

The reversible production of plateaus in intestinal muscle emphasizes the basic similarity of smooth muscle to many other excitable tissues.

Summary. Sucrose gap records from strips of cat circular intestinal muscle show under influence of barium a reversible transformation of spikes to plateaus lasting many seconds and topped by oscillations. Acetylcholine on cat longitudinal intestinal muscle may induce either rhythmic plateaus topped by spikes or a maintained depolarization, depending on concentration.

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Received November 23, 1959. P.S.E.B.M., 1960, v103.

Fluorescence of Bone after Quercetin Ingestion. (25485)

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Preferential deposition of many substances in bone is well-known, but induced fluorescence of bone is uncommon, and to our knowledge has been reported only for tetracycline antibiotics(1). The present report describes the phenomenon in quercetin-fed rats of fluorescence of excised bone when viewed under ultraviolet light (UV). Quercetin (3,5,7,3′,4′ pentahydroxyflavone) is widely distributed in the plant kingdom as either glycoside or aglycone(2). It exhibits weak dull-orange fluorescence under UV. The metabolic fate of quercetin has been described(3,4,5). One of its, as yet unidentified, urinary metabolites exhibits brilliant golden-yellow fluorescence under UV. Lack of toxicity of quercetin has been reported(6).

Methods. Quercetin is commercially available, but the present product was prepared by acid hydrolysis of its rhamnoside, quercitrin, isolated from lemon flavine(7) a commercial dyestuff derived from bark of black oak tree. Quercetin was mixed at level of 1% in diet having the following percentage composition: degerminated yellow corn meal 73, casein 10, linseed oil cake meal 10, ground alfalfa 2, bone ash 1.5, sodium chloride 0.5, and cod liver oil 3. Groups of weanling, adolescent, and adult albino rats from our colony were fed the quercetin-containing diet for various periods, then sacrificed and the excised bones examined grossly, under UV, and microscopically.

Results. When 21-day old rats were fed quercetin-containing diet for 3 days their bones had an intense golden-yellow fluorescence under UV. Maximum fluorescence was apparently achieved by 10th day of feeding. Fluorescence persisted at least 6 weeks after withholding quercetin from the diet. Persistence of fluorescence for longer periods was not studied. Color and intensity of fluorescence appeared similar to that described for rats and rabbits receiving tetracycline antibiotics(8). Rats fed quercetin did not differ from controls in growth and development(6,9), or in gross and microscopic bone structure. Some, if not all, fluorescence persisted after extraction of bone calcium with 5% nitric acid for 3 days. Fluorescence was lost in preparation of paraffin sections. It diffused slowly from bony tissue into formalin solutions when allowed to stand for several weeks. Fluorescence was not seen in non-osseous tissues. Comparable experiments with adolescent and adult rats showed fluorescence was least in old rats and intermediate in adolescents.

Discussion. The nature of the fluorescent substance in bone and mode of its deposition are yet to be elucidated. Its possible identity with the fluorescent urinary metabolite of quercetin merits consideration. The greater concentration in young growing bone suggests a relationship to bone growth rather than mere deposition. Persistence after decalcification of bone suggests a combination other than simple chelation.

The value of induced bone fluorescence for autophotographic studies of bone development, dyscrasias, and neoplasms is obvious. It may be applicable in endocrine studies for assay of growth hormone by measurements of the epiphyseal plate.

Summary. Feeding 1% quercetin in the diet induces fluorescence in bones of rats when such bones are viewed under ultraviolet light. Fluorescence appears the third day of feeding and persists at least 6 weeks after cessation of feeding. Nature of fluorescent substance and mode of its deposition are not clear. Magnitude of fluorescence appears to be related to bone growth. Possible uses of this phenomenon are suggested.

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Received November 24, 1959. P.S.E.B.M., 1960, v103.

Constitution of Bile Proteins.* (25486)

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It is unlikely that the gallstone problem will be completely resolved before the biochemistry and physiology of bile are thoroughly understood. Unfortunately little is known about the nature of bile proteins even though several investigations concerning bile proteins have been reported(1,2,3,4,5,6). In this study various bile protein fractions are identified, classified and further characterized with respect to their physico-chemical properties.

Materials and methods. Female sheep weighing 60 to 100 lb and dogs weighing 40 to 50 lb were used. Gallbladder bile was obtained by puncture of gallbladder followed by cholecystectomy. Hepatic bile was obtained from T-tube inserted into the common duct. The long end of the T-tube was plugged with a glass rod and fixed under the skin between bile collections. Subsequent collections of bile from the T-tube were made by incising the skin under pentobarbital anesthesia and connecting a plastic tube to the long end of the T-tube. Bile samples were concentrated to approximatey 1/10 vol. by dialysis with the use of 50% (w/v) gum arabic solution in barbital buffer (pH 8.6, ionic strength 0.1)(7). Paper electrophoresis of concentrated bile samples was performed in barbital buffer of ionic strength 0.1, pH 8.6 for 15 hours at 200 volts and at 8°C using horizontal strip type apparatus(8). Approximately 0.1 ml of concentrated bile was used on filter paper strip (4.5 x 34.0 cm) of Whatman 3 MM filter paper. Since bile pigments and phospholipids are also stained with bromphenol blue and interfere with quantitative evaluation of bile protein fractions, filter paper strips were washed overnight in a mixture of ethanol: chloroform : concentrated hydrochloric acid (2:1:0.01). Proteins on filter paper strip were then stained with bromphenol blue(9). The stained filter paper strips were immersed into melted paraffin at approximately 60°C and color intensity measured with Photovolt densitometer model 525, using a filter of 570 mμ. The faster migrating A-fraction (see Results) was isolated electrophoretically as follows: A small amount of buffer was placed in a cellophane bag set in the anodal buffer vessel of paper electrophoresis apparatus. The anodal end of filter paper strip was placed in this buffer and the A-fraction was allowed to migrate into this buffer by means of electrophoresis and collected. Buffer was removed from the A-fraction as much as possible by dialysis and electrodialysis, then the A-fraction was concentrated by the ultrafiltration method of Mies(10). For paper chromatographic analysis of amino acids, the A-fraction was hydrolyzed in a sealed glass tube with 8 N hydrochloric acid at 100°C for 24 hours. An n-butanol : acetic acid : water

* This investigation supported by grants from Nat. Inst. Health, Research Corp. of Detroit Receiving Hospital, and Parke, Davis & Co.

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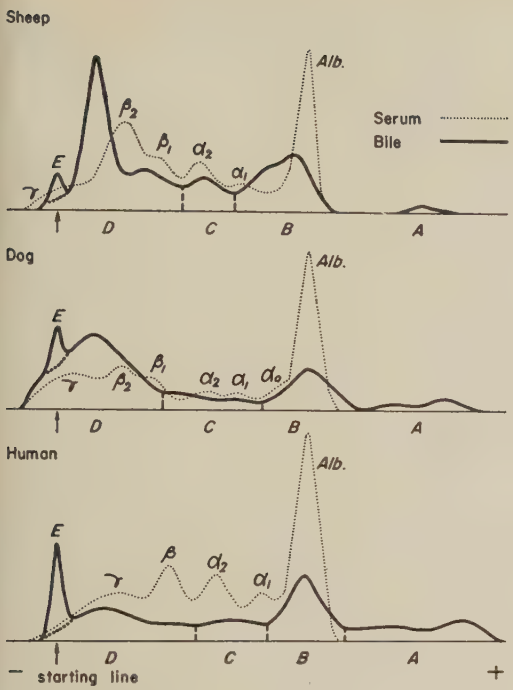

FIG. 1. Comparison of hepatic bile and serum protein fractions.

mixture (4:1:5) was used as solvent. A 0.2% solution of ninhydrin in water-saturated *n*-butanol was used to detect amino acids. For paper chromatographic analysis of carbohydrates, the A-fraction was hydrolyzed under reflux with 2 N hydrochloric acid for 12 hours. A phenol:water mixture (4:1) was used as solvent. An aniline hydrogen phthalate reagent was used to detect carbohydrates.

Results. The curves obtained from paper electrophoresis of the concentrated hepatic bile and serum are shown in Fig. 1. The paper electrophoretic patterns of bile and serum proteins differed among species, corresponding protein peaks were always present.

1. *Classification of bile proteins.* The bile curve might be divided into A-, B-, C-, D-, and E-fractions (Fig. 1). B-, C-, and D-fractions consisted of those proteins which migrated the same as serum protein fractions. The A-fraction migrated more rapidly than any protein fractions in serum. The E-fraction remained on starting line. Normal bile and bile from patients with gallstones contained all of these fractions.

2. *Nature of A- and E-fractions of bile proteins.* A- and E-fractions were unique to bile insofar as no comparable fractions were seen in serum (Fig. 1). The A-fraction was present in fresh bile in significant amounts, diminishing after concentration of the bile. Conversely, the E-fraction was not prominent in electrophoresis of fresh bile but became prominent in electrophoresis of concentrated bile. The E-fraction was insoluble in barbital buffer (pH 8.6, ionic strength 0.1), so that it did not migrate and remained on the starting line during electrophoresis. The E-fraction, as well as the A-fraction, did not combine with bromphenol blue at pH 8.6, but combined with bile pigments. From these facts, it was assumed that the E-fraction consisted of proteins of the A-fraction denatured in process of concentration.

Paper chromatography of the hydrolysate of the A-fraction separated as described under Methods, revealed the presence of various amino acids, hexose and hexosamine. The black precipitate remaining after hydrolysis was readily soluble in anhydrous ethanol, ether and chloroform. It was slightly soluble in 50% ethanol and was insoluble in water. The A-fraction was stained with Sudan black B. The precipitate therefore was assumed to be composed mainly of lipids. Furthermore, the A-fraction was not heat coagulable. It was therefore assumed that the A-fraction

TABLE I. Constitution of Bile Proteins.

	Type of bile	No. of cases	Bile protein fractions (%)* A	B	C	D	E
Sheep	Gallbladder bile	6	2.1 ± 1.7	42.8 ± 1.4	25.3 ± 4.3	25.3 ± 4.9	4.5 ± 2.9
	Hepatic "	4	2.8 ± 1.8	42.4 ± 2.1	14.0 ± 2.1	36.2 ± 6.5	4.6 ± 2.1
Dog	Gallbladder "	6	4.6 ± 1.9	5.1 ± 3.0	6.1 ± 2.1	72.0 ± 5.9	12.3 ± 4.1
	Hepatic "	5	15.7 ± 2.7	17.8 ± 5.2	8.3 ± 2.8	54.5 ± 4.3	3.8 ± 1.6
Human	Gallbladder " †	1	43.0	35.3	4.4	15.5	1.8

* Mean ± S.D. (except human gallbladder bile). † Cholelithiasis.

contained a mucoprotein and a lipoprotein.

3. *Nature of B-, C- and D-fractions.* Unlike the A- and E-fractions, mobilities of B-, C- and D-fractions were comparable to serum protein fractions, and were thus assumed to be similar to the serum proteins.

The B-fraction migrated at a rate similar to that of serum albumin. However, the evidence for this similarity also came from other sources than their similar mobilities. For instance, it was reported that bilirubin in serum was combined with albumin and α-globulin (12,13,14,15) and bile pigments likewise combined with the B-fraction. Furthermore, Kunkel and Tiselius(9) reported that bromphenol blue combined only with serum albumin when it was added to serum prior to electrophoresis at pH 8.6. Similarly, bromphenol blue migrated with the B-fraction when it was added to bile prior to electrophoresis at pH 8.6.

Summary. 1. Proteins detectable by paper electrophoresis of concentrated bile were classified into A-, B-, C-, D- and E-fractions. 2. The A-fraction migrated more rapidly than any protein fractions in serum. 3. The B-fraction was similar to serum albumin as evidenced by its similar mobility to serum albumin and affinity for bile pigments and bromphenol blue. 4. C- and D-fractions had mobilities similar to serum globulins. 5. The E-fraction was probably a denatured A-fraction, and became prominent only after concentration of bile, associated with a decrease in the A-fraction. 6. Normal and pathological human bile, the bile of sheep and dog contain all these protein fractions. 7. Values of bile protein fractions varied considerably according to species and type of bile.

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Received July 6, 1959. P.S.E.B.M., 1960, v103.

ACTH Releasing Factor Active in the Guinea Pig.* (25487)

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The hypothalamus produces a chemical mediator concerned with ACTH release(1). It is presumably transported by way of neurohypophyseal tracts into the posterior pituitary gland where it is stored(2). There is now much evidence which points to presence of a substance in extracts of posterior pituitary that is capable of causing ACTH release(3,4, 5); apparently this is related to the hypothalamic agent. Certain investigations lead to the belief that this substance is vasopressin (6,7,8,9). However, powerful evidence suggests that it differs from vasopressin(10,11, 12). This communication presents the results which demonstrate that vasopressin

* This project was supported by grant from U.S.P.H.S.

† This investigation was carried out in partial fulfillment for requirements of degree of Doctor of Philosophy.

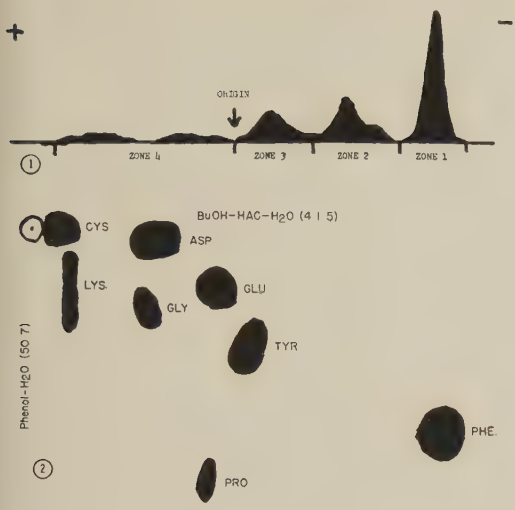

FIG. 1. Commercial pitressin powder fractionated by electrophoresis at pH 5.7, and 0.1 M acetate buffer. 100 volts for 17 hr in Spinco paper electrophoresis apparatus. Stained by spraying with 0.1% ninhydrin, collidine and acetic acid in alcohol(17). The pattern was obtained with the Spinco Analytrol.

FIG. 2. Amino acid and composition of material in zone 1.

causes ACTH release in the guinea pig.

Methods. The procedure for measuring ACTH release depends upon increase in corticoid excretion by the guinea pig(13,3). Young guinea pigs (300-450 g) of either sex were used. Urine was expressed from bladder and the material for testing was injected intraperitoneally in 5 cc of saline. Blocking agents were not used since it has already been demonstrated that pitressin is active in causing increased corticoid excretion in presence of morphine(14). Animals were placed in urine collection cages for 6 hours. Bladders were again emptied and specimens frozen until used. Corticoids were determined by procedure of Silber and Porter(15). Pressor activity was determined on cats anesthetized with nembutal and in which the carotid artery was catheterized. All injections were given into femoral vein in saline and followed by saline washes. A recovery period of 30 minutes was allowed before the next injection. Recordings were made on smoked drum kymograph. The pressor test was used rather than ADH assay because following thiosorbitol treatment according to procedure of Croxatto(16), pressor and ACTH releasing activities are destroyed while ADH activity is retained.‡

Results. Commercial pitressin powder§ was subjected to electrophoresis at pH 5.7 in 0.1 M acetate buffer according to conditions developed by Taylor *et al.*(17). Four zones were obtained (Fig. 1). The pattern appears identical with that obtained by Taylor *et al.* (17) with similar material obtained from beef. The foremost cathodic zone (fraction 1) had a mobility which suggested that the material was vasopressin(17). Vasopressin is unique in that its isoelectric point is 10.9 which makes it one of the most basic natural substances known(17).

For preparative work sheets of Whatman #3 mm, 12½ x 11¼ inches were used and 20 mg of commercial powder was applied across the sheet. The zones were identified by staining of lateral strips. Individual zones were eluted with 0.25% acetic acid. Resulting solutions were dried by lyophilization and the products, which contained in excess of 97% of salt, were used without further purification. The material from each fraction was assayed for ACTH releasing activity and pressor activity (Table I). Only zone 1 was active in these tests.

The amino acid composition of zone 1 was determined as follows: The lyophilized ma-

TABLE I. Activity of Fractions Obtained by Electrophoresis.

	No.	Guinea pig corticoid excr., μg/6 hr	Cat pressor response increase, mm Hg
Control	18	75 ± 10*	
Zone 1	18	173 ± 22[1]	28[2]
2	19	77 ± 13[1]	8[3]
3	19	66 ± 9[1]	0[4]
4	18	66 ± 11[1]	0[5]

[1] 100 μg of lyophilized material * Stand. error
[2] 40 *Idem*
[3] 280 "
[4] 280 "
[5] 350 "

‡ Sobel, H., and Schapiro, S., unpublished data.

§ Obtained through generosity of Dr. D. A. McGinty of Parke, Davis & Co. This material assayed 70 U/mg. Commercial pitressin may be of bovine (arginine vasopressin) or porcine (lysine vasopressin) origin. Pitressin refers to commercial mixture, vasopressin to isolated peptide.

terial was heated in sealed tubes for 6 to 8 hours with 0.02 cc of constant boiling H Cl/mg at 150°C. The hydrolysate was chromotographed on Whatman #1 paper according to procedure of Levy and Chung(18) except that the phenolic phase contained no m-cresol. Only 8 amino acids were detected and the material corresponded in composition with that of lysine vasopressin (Fig. 2).

It has been shown that mild acid hydrolysis of pitressin causes loss of ACTH releasing activity‖(3). A study was made of the effect of mild alkaline and acid treatment upon pressor and ACTH releasing activity of the material in zone 1. After numerous trials, the conditions employed were as follows: Material from the active zone was dissolved in solution of HCl (pH 1.2) and NaOH (pH 11.55). The solutions were placed in boiling water bath for following times: in acid 10, 20 and 30 minutes, in alkali 5, 10 and 20 minutes. After the required intervals the solutions were immediately placed in ice water and pH adjusted to 7.4 for injection. The results are shown in Table II. Under both conditions decrease in ACTH releasing activity was paralleled by decrease in pressor activity.

The effect of iodination was investigated. A solution of iodine in KI was added to pitressin in acetate buffer pH 4.6 and phosphate buffer pH 7.4 until a brownish tinge remained after stirring. The solution was placed in refrigerator 1 hour to complete iodination. The solution in acetate buffer was brought to 7.4 before injection. Both ACTH releasing and pressor activities were destroyed.

Placenta is capable of destroying pressor activity of pitressin(21). A study was made of the effect upon ACTH releasing activity.

TABLE II. Effect of Treatment upon ACTH Releasing and Pressor Activity of Material from Zone 1 and Pitressin.

	No.	Urinary corticoids, μg/6 hr	ΔC	ΔP	ΔC/ΔP
Control	13	62 ± 5*			
Hydrolysis pH 1.2					
0 min.	9	126† ± 16	64	38¶	1.7
10	8	121 ± 19	59	32	1.5
20	8	102 ± 15	40	26	1.5
30	7	97 ± 13	35	23	1.5
Hydrolysis pH 11.55					
0 min.	8	122† ± 15	60	21**	2.9
5	9	120 ± 21	58	20	"
10	8	103 ± 15	41	14	"
20	9	80 ± 9	18	7	2.6
Control	10	62 ± 9			
Pitressin (pH 4.6)	10	163‡ ± 11			
Idem, iodinated	11	68 ± 9			
Control	9	48 ± 12			
Pitressin (pH 7.4)	9	125 ± 21			
Idem, iodinated	9	63 ± 9			
Control	4	81 ± 8			
Pitressin	5	266§ ± 16			
Placental extract‖	5	91 ± 20			
Idem + pitressin	5	73 ± 19			
Boiled extract + pitressin	5	323 ± 30			

ΔC = Difference from control corticoid value.
ΔP = *Idem* blood pressure.
* Stand. error.
† 100 μg of fraction I/100 g body wt.
‡ 1 unit pitressin/100 g body wt.
§ 2 units *Idem* .
‖ Placental extract placed after incubation in boiling water bath for 3 min. Denatured protein removed.
¶ .058 mg fraction I.
** .048 *Idem* .

An extract was made from fresh uninfarcted placental tissue according to procedure of Hawker(21). Pitressin was incubated with the extract for 2 hours. This treatment is known to inactivate pressor activity. ACTH releasing activity was lost.

Discussion. Our findings suggest that lysine vasopressin or another substance very similar to it in composition and stability may cause ACTH to be released by the guinea pig. The latter (remote) possibility must be considered since only simple electrophoretic separation was used. This requires the existence of a substance of similar electrophoretic behavior at pH 5.7, a highly basic polypeptide which responds similarly to partial hydrolysis at pH 1.2 and pH 11.55, to iodination at pH

‖ It has been suggested that vasopressin does not act through ACTH release but rather that it acts directly upon adrenals(19). The conclusion that this may be the mode of action of pitressin is inconsistent with previous findings that pitressin is not effective in hypophysectomized guinea pigs(3). Furthermore other substances, e.g. serotonin (20) may act directly upon the adrenal. It is possible that direct actions are due to vascular phenomena. The evidence now seems overwhelmingly in favor of an ACTH releasing mechanism.

4.6 and pH 7.4, to placental extract and thiosorbital treatment. It is possible that an amide grouping on the glycine moiety accounts for the lability to dilute acid as it does in oxytocin(22).

Several investigators prepared a pressor-free substance from extracts of posterior pituitary(10,11). The preparation of Schally *et. al*(11) contains 10 amino acids including alanine, serine and histadine, in addition to those present in lysine vasopressin, but excluding tyrosine. Furthermore the substance is remarkably resistant to acid hydrolysis. This suggests that this substance is similar to lysine vasopressin, differing from it only in that histidine has replaced tyrosine in the ring and alanine and serine have replaced amide groups. This latter replacement would make the molecule much more acid resistant.

Guillemin *et al.*(23) report that 40 μg of their fraction D, a crude preparation which contains 1/100 of its weight of fraction D-delta (the active component) causes a corticoid response in the rat, equal to that produced by 128 mU (.44 μg) of their purified lysine vasopressin. Although a pressor-free substance exists which is capable of increasing ACTH release, the physiological significance of vasopressin in this regard must not be ignored. It is proposed that in an anatomical system like the portal veins a non-pressor amount of vasopressin might activate ACTH release.

Summary. Lysine vasopressin was obtained from commercial pitressin powder by electrophoretic separation. The ACTH releasing activity in the guinea pig and the pressor activity in the cat were compared following various chemical manipulations. Following mild acid and alkaline hydrolysis, iodination, and incubation with placental extract both activities are altered to the same extent. It was concluded that lysine vasopressin causes ACTH release in the guinea pig. This is consistent with the concept that it may be a hypothalamic mediator in this species.

The authors are grateful to Dr. H. Bergman for the presser assays.

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Received July 22, 1959. P.S.E.B.M., 1960, v103.

Effect of Methyl Bis (2-Chlorethyl) Amine Upon Survival of Skin Homografts in Rats and Rabbits.* (25488)

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Survival of homografted tissues has been shown to be possible where the immune response of host is congenitally impaired(1) or destroyed by X-irradiation(2,3). Chlorethylamine compounds, of which nitrogen mustard (HN_2) is representative, have a depressant and destructive effect upon bone marrow and lymphatic tissues. Hektoen and Corpor(4), studying sulfur mustards, first described an effect of this class of compounds upon immune mechanisms. More recently, Phillips and coworkers(5), Spurr(6), Bukantz *et al.*(7), and Schwab and associates(8) described a depression of titers of the antibody response to bacterial antigens and a delay in appearance of circulating antibodies after administration of nitrogen mustard. Recent studies by Green (9) question the degree of effect of nitrogen mustard upon antibody titers ultimately achieved. However, the profound change in lymphoid tissues after administering HN_2 suggest that there may be a hampering effect upon host organism's reactive capacity to homografted tissues. Furthermore, the role of circulating cells in homograft rejection response would indicate that a profound depression of circulating cellular elements should affect homograft survival. Infiltration of a homograft with cells, presumably from host circulation, is a prominent morphological accompaniment of rejection. On the other hand the classic experiments of Algire, Weaver, and Prehn(10), with millipore membranes interposed between host and graft, demonstrated that total exclusion of host cells allowed homograft survival. We were, therefore, attracted by the report of Levinson and Necheles(11), which suggested that a marked prolongation of homograft survival occurred in rats as a result of treatment with relatively low doses of nitrogen mustard. The following study was therefore carried out.

Methods. In 2 experiments, female rats (2 varieties of Sprague-Dawley origin) from Holtzman Co. and from Rolfsmeyer Co. were used. Female Long-Evans rats from Rockland Farms were a third strain used. Rats weighed 150-175 g each. Rats were equally distributed between treatment and control groups. In all instances reciprocal grafts were exchanged between treatment and control groups. The second group consisted of intrastrain grafts between Holtzman rats. Dosage schedules are summarized in Table I. In Group II both strain combination and dosage were selected to duplicate methods reported by Levinson and Necheles(11). All solutions of methyl bis (2-chlorethyl) amine (Mustargen‡) (HN2) were freshly prepared and injected into a tail vein immediately after dilution with appropriate volume of saline. In all grafting procedures, control and treatment group animals were selected by chance and lightly anesthetized with ether. 9 cm^2 full thickness skin grafts were removed from ventral thorax and abdomen by careful dissection in the fascial plane superficial to the panniculus carnosus and the recipient site was prepared in a similar fashion. Homografts were exchanged between pairs of animals by reciprocally exchanging skin from abdominal donor site of one animal and suturing it in the thoracic recipient site of the other animal. Skin from the thoracic donor site was sutured in the abdominal recipient bed of the same animal to serve as an autograft for evaluation of infection and technic. Care was taken to reverse the hair direction for future identification of the graft site. All grafts were sutured with interrupted 6-0 silk sutures at the corners and the continuous 6-0 silk suture

* Supported by grants from USPHS, Am. Heart Assn., Minn. Heart Assn., and Graduate School of Univ. of Minnesota.

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‡Generously supplied by Dr. Nicholas Capeci of Merck Sharp and Dohme.

TABLE I. Effects of Nitrogen Mustard on Rat Skin Homografts.

Group	Strains	No. treated	Dosage schedule	Wt loss	Leuko-penia	Effect on homograft
I	Rolfsmeyer Long-Evans	10	1 mg/kg on days 1, 3, 5, post-grafting only	++	±	Grafts rejected at same time as controls*
II	Holtzman intra-strain grafts	20	4 mg/kg on 1st day post-grafting and every 4 days for 7 doses	+		

* Sufficient quantitative data to make a statistically valid statement at 95% probability level.

around the margin. A thin layer of Bacitracin® ointment was applied to wound edges after which the graft was covered with a protective dressing consisting of a gauze pad held in place with several turns of waterproof adhesive tape. Dressings were changed every 2 days as long as grafts persisted. Daily examination of each graft was made possible by splitting the bandage along the back and retaping. In grafts between Long-Evans and Rolfsmeyer Sprague-Dawley rats, onset of rejection in untreated animals appeared abruptly and was a distinct endpoint revealed in cessation of capillary circulation as described by Taylor and Lehfeld(12). In Group II, intrastrain Holtzman-to-Holtzman grafts, onset of rejection typically was quite subtle and attenuated. Therefore, it was necessary to utilize another, less satisfactory standard for determining survival status in these sites of delayed rejection. We concluded that the grafts were no longer viable after a distinct follicular pattern became indiscernible because the donor epithelium was no longer identifiable under the microscope as revealed in serial biopsies made during this time sequence. Autopsies were performed and electrophoretic studies of serum were made on 5 additional rats treated according to dosage schedule used in Group II. Lymphatic tissues were grossly examined in representative animals of the other groups. In studies using rabbits, nitrogen mustard was given *via* ear vein. In all instances, grafting was reciprocally done between albino rabbits and black-and-white Dutch rabbits, both varieties obtained from commercial sources. During period of treatment with nitrogen mustard, daily white counts were done on all treated animals. The grafting procedure in rabbits differed from that in rats in the following ways: Pairs of animals were anesthetized with I.V. Nembutal, grafts were larger (12 cm^2), and protective dressing consisted of several turns of rapid-drying plaster of paris bandage over a gauze pad. These too were split down the back and retaped for daily inspection. Three studies were conducted in rabbits. *First,* 8 of the albino strain were intensively pretreated with 1 mg/kg of HN_2 for 4 days. On fifth day, when marked leukopenia was evident grafts were exchanged between treated animals and 8 Dutch rabbits. Additional doses of HN_2 were administered to all treated animals whenever the leukocyte count tended to rise. The days HN_2 was given are indicated by a solid bar at bottom of Fig. 1. *Second,* grafts were reciprocally exchanged between 13 albino rabbits and 13 Dutch rabbits. The 2 varieties were distributed equally as to treatment and control groups, one member of each pair being in the treatment group. HN_2 in doses of 1 mg/kg, was administered to the treatment group on day of grafting and for each of the next 3 days. Additional doses of 1 mg/kg were given on an individual basis, as needed to maintain leukocyte count below 2000 cells/mm^3. Relative height of solid bars on the baseline in Fig. 2 denotes number of animals receiving HN_2. *Third,* grafts were exchanged between 16 albino and 16 Dutch rabbits. One member of each reciprocally grafted pair was allocated to the treatment group, giving equal distribution of the 2 varieties of rabbits to treatment and control groups. Beginning 5 days following grafting, 2 mg/kg of HN_2 was administered to each rabbit daily for 3 days. Thereafter, the same dose was administered on an individual basis as required to maintain leukopenia. Relative height of bars in Fig. 3 indicates number of animals receiving HN_2. In all 3 treatment

groups, daily leukocyte counts were done on each animal. Differential leukocyte counts were done on treated rabbits in Group 1. Weights were taken serially. Penicillin and streptomycin were given every 2 days to combat development of respiratory infection.

Results. Dosage schedules, strain combinations, and effect of nitrogen mustard upon weight, total number of leukocytes, and homograft survival in rats are summarized in Table I. All rats in Group 1 had weight loss following HN_2 averaging 44 g/animal but the presence of leukopenia was variable from animal to animal. Grafts in the treatment group survived a mean of 9.2 days compared to a mean of 8 days in untreated controls. A "t" test confirms the lack of a significant difference in survival times. A 50% mortality resulted from HN_2 treatment whereas all 10 control animals survived well beyond the homograft slough time.

The experiment in "Group II rats" uses animals from the same source and repeats dosage schedule reported by Levinson and Necheles. No mortality resulted from this dosage schedule. In the treated group, total leukocyte count remained within pretreatment limits with the exception of 2 animals showing a transient leukopenia. In other experiments it was impossible to produce significant leukopenia with this dosage schedule, even though histologic study shows marked bone marrow suppression, lymph node and splenic atrophy. Weight loss averagd 43 g/animal in 8 animals, 2 animals lost no weight. Mean homograft survival in Group II was 27.3 days and 26.2 days in treatment and control groups respectively. This difference is not significant ($P > 0.50$). The longest graft survivals, over 100 days, occurred in 3 untreated control animals and 2 treated animals. The grafts had good hair regrowth and appeared normal except for altered hair direction. These prolongations were entered in the computations as 57 day survivals, the longest survival of any graft which was ultimately rejected. Animals treated according to the schedule of Group II had only a mild depression of the alpha-1 globulins on electrophoretic analysis.

Results of experiments in rabbits are summarized in Fig. 1, 2, and 3. In all 3 groups,

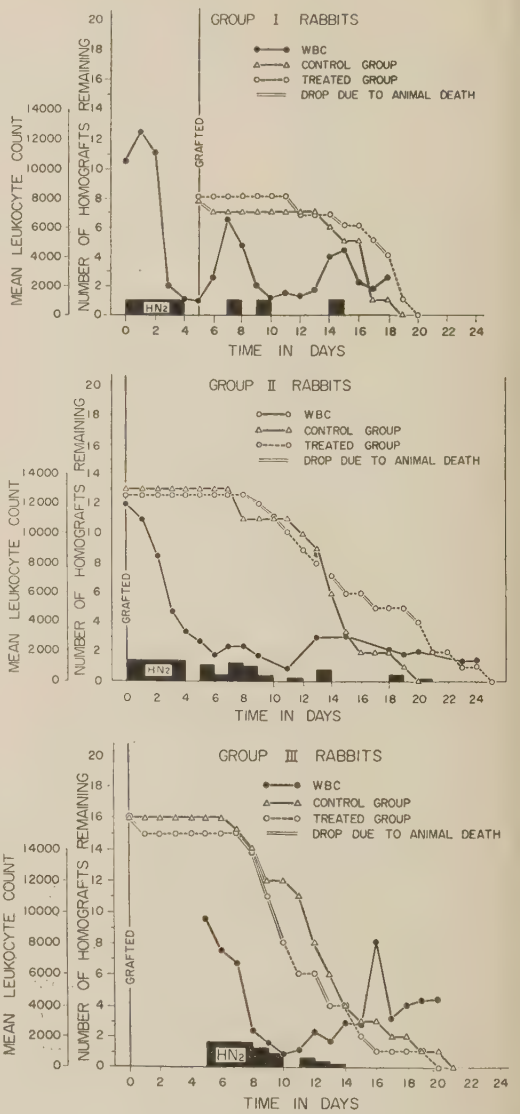

FIG. 1-3.

regardless of treatment used, certain general statements apply. *First,* a prolonged leukopenia could be produced. *Second,* treatment with HN_2 produced a significant mortality but survival was adequate to allow some quantitation of effect of treatment on skin homograft survival. *Third,* weight loss and anorexia were universal in treated rabbits.

The pretreated rabbits in Group 1 (Fig. 1) had a mean leukocyte count at time of placement of grafts of about 3000 cells/mm^3. This average value includes the 2 short periods of

WBC rebound. However, this preliminary depression of the reticular system resulting from intensive HN_2 treatment did not influence ultimate survival of grafts or alter time sequence of graft rejection. In Group 2 rabbits (Fig. 2), the more intense individualized treatment resulted in a more marked leukopenia and a significantly increased mortality. Although 2 grafts in the treated group survived longer than any of the control grafts, the prolongation was slight and not statistically significant (does not approach 90% probability). In Group 3 (Fig. 3) where HN_2 treatment was withheld until 5 days after grafting when vascular continuity between graft and host can be assumed to have been established, vigorous treatment produced a marked leukopenia, but was ineffectual in altering graft rejection since no graft in the treatment group survived longer than in controls.

In all 3 groups of rabbits, treated or controls, the endpoint of homograft rejection was clear-cut and abrupt. In 95% of all animals, the control autografts survived, appeared normal and in all animals surviving, the native transplant developed a regrowth of hair. In these rabbits, autopsies revealed lymph nodes which were small and atrophic. The differential leukocyte counts in Group I had that pattern essentially as described by Stetson and Good(13) for these dosages of HN_2 in rabbits.

Discussion. Our experiments emphasize that intensive HN_2 treatment does not significantly prolong survival of skin homografts when administered before, at the time of, or following grafting in rats and rabbits of these strains. This is true even with marked effect of treatment upon lymphoid tissues. This would seem to indicate that only a fraction of the antibody response mechanisms need to be functionally intact to prejudice survival of a homograft. Even HN_2 doses which proved fatal over 15-20 days produced, at most, but slight temporal delay in loss of homograft integrity. If one accepts the plasma cell system as being the principal center of antibody formation, the observations of Marshall and White(14) that the plasma cell system has proven relatively resistant to effects of nitrogen mustard would explain our failure to achieve depression of host immunity to the point of tolerance for a homograft. Furthermore, a recent publication by Green(9), appearing after this study was undertaken, described temporal relationships which condition the apparent effects of nitrogen mustard on antibody formation as revealed by timing of sampling technics following HN_2 administration. Green stimulated antibody production in rabbits by administering typhoid toxoid intravenously at varying intervals before, during and after 4 daily doses of HN_2 in 1 mg/kg doses. He then followed the titers of antibody in a serial manner. Maximum elevations in antibody titer were observed when the antigen was given after the second day of nitrogen mustard treatment. The antigen then elicited a delayed production of antibody, but titers ultimately equaled those of untreated control animals. When antibody appearance was maximally delayed, the peak occurred at about 15 days after toxoid stimulation rather than the 5 to 8 day peak titer noted in controls. Inherently the experimental design of earlier studies of effect of HN_2 on antibody production concealed the differing times for peak response in antibody formation.

These studies of homograft survival suggest existence of a lag in the host rejection mechanism. If the delay in transfer of antigenic material from the homograft to the host is about 24 to 48 hours, as suggested by data of Converse and co-workers(15), the work of Green (9) would indicate that the HN_2 therapy in Group II rabbits should have resulted in a maximal delay in rejection. If antibodies to histocompatibility factors are produced in the same time sequence as antibodies to a bacterial antigen, antibodies of histocompatibility antigens would occur soon enough to prevent significant prolongation of homograft survival. However, since animals receiving effective HN_2 doses all had weight loss and cachexia, any small prolongation in survival could represent a nutritional or metabolic effect rather than a primary effect of nitrogen mustard upon antibody producing cells.

The fact that marked, prolonged suppression of circulating leukocytes failed to prolong survival of skin homografts is of interest. This further supports the conclusion that if pro-

longation of survival of homografts is to be attempted by an attack on the cellular factors of rejection, exclusion of host cells must be virtually absolute either by chemically induced leukopenia or by interposing a suitable millipore filter between host and graft(10).

Finally, we must explain the conflict of our findings with those of Levinson and Necheles (11), who reported prolongations of graft survival up to 114 days by administering nitrogen mustard in doses of 0.4 mg/kg every 4 to 6 days for 40 days. The fact that employing their dosage schedule effect no change in outcome of intrastrain homografts (Group IV, Table I) when HN_2 treated animals were compared with simultaneous controls speaks against any profound effect of HN_2 on homograft survival. Certainly, this is the most favorable experimental design with a minimum of factors working against homograft acceptance. The key to the conflict appears to lie in the statement of Levinson and Necheles that "Sprague-Dawley Holtzman rats were utilized" presumably as both donor and recipient. We prevously reported the fact that frequently permanent survivals occur with intrastrain grafts in these inbred animals from this particular source(16). Since the prolongations reported by Levinson and Necheles do not exceed the survival found in reciprocal grafts between untreated Holtzman Sprague-Dawley rats, we can only conclude that prolongation of homograft survival reported by them was due to their selection of experimental animals and the absence of an adequate pairing between reciprocally grafted controls.

Summary. Intensive administration of methyl bis (2 chlorethyl) amine (Nitrogen mustard) to rats and rabbits in doses sufficient to produce and maintain marked leukopenia did not depress host response mechanisms in a degree sufficient to allow significant prolongation of homograft survival.

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Received July 27, 1959. P.S.E.B.M., 1960, v103.

Effect of L-3,3′,5-Triiodothyronine on Epinephrine-Induced Contraction of Isolated Rabbit Aortic Strip. (25489)

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Thyroxine has been reported to increase sensitivity of isolated perfused swine arteries to epinephrine as evidenced by increase in degree and duration of constriction(1). Increased sensitivity to epinephrine was attributed to inhibition of amine oxidase in walls of the artery. Other experiments* indicated

* Personal communication from R. A. McLean and R. J. Geus of Smith Kline & French Labs., Phila., Pa.

that L-3,3′,5-Triiodothyronine (T_3), a thyromimetic agent closely related to thyroxine, potentiates epinephrine-induced contraction of another vascular smooth muscle, that of isolated rabbit aortic strip. Results of several investigations(2) indicated that ability of various agents to potentiate the effects of epinephrine on isolated organs can quite often be related to chelating or complexing action of these compounds. The purpose of our study was to determine if a similar metal-dependent mechanism was operating in *in vitro* potentiation of epinephrine by T_3.

Method. Spiral strips of rabbit aorta were mounted in 50 ml muscle chambers according to the method described by Furchgott(3). The muscle bath, maintained at 37°C, contained a modified Ringer's solution with 1 g of glucose/liter. 95% O_2-5% CO_2 was bubbled through both muscle bath and reservoir bottle, giving pH 7.4. Contractions of aortic strips were recorded on smoked kymograph by isotonic levers. Distilled, demineralized water was used in making Ringer's and isotonic saline. The stock solution of l-epinephrine hydrochloride (Parke-Davis) was kept frozen prior to use. Working solutions of desired concentration were freshly prepared by triturating the drug in isotonic saline, adjusting the pH to 8.5-9.0 with 0.1N NaOH and stiring until all drug was in solution. Isotonic saline served as vehicle for all other drugs used. The volume of any drug addition to muscle bath was 0.2 ml or less. Drug concentrations refer to the salt except for T_3, where concentration is expressed in terms of free acid.

Results. 1) *Control* responses to standard 1×10^{-7} concentration of epinephrine were determined only after 2½ to 3 hours following the mounting of aortic strips in muscle chamber. According to Furchgott(3), maximum sensitivity to epinephrine is reached at this time, after which the contractile response to epinephrine does not significantly change. This finding was confirmed in the present study. Control responses to epinephrine were of 2 types. In most experiments, epinephrine-induced contractions were quite prolonged, often lasting 1½ hours or more. In a few experiments, however, duration of epinephrine contraction was relatively short (10 to 30 minutes). The height of contraction averaged about 10 mm in both cases, which was well below the maximum possible.

2) *Effect of T_3 on Epinephrine-induced Contractions.* After 2 or 3 epinephrine control responses were observed, with washing after each, T_3 was injected into the bath to a concentration of 1×10^{-6}. No response was evoked in any experiment by T_3 alone. The contractile response to epinephrine was again recorded one-half hour later, and at least once more after this. The bath was not washed during this period. T_3 failed to enhance significantly the epinephrine-induced contraction, in height, in experiments in which the control response was of long duration (Table I). Determination of possible potentiation in terms of duration was not feasible because of quite prolonged control responses. When control response was of short duration, T_3 significantly potentiated the epinephrine-induced contraction in height and particularly in duration. When copper acetate was added to the bath (2×10^{-7}), the usually prolonged contractions after epinephrine were considerably shortened. T_3 again significantly potentiated epinephrine-induced contraction.

3) *Effect of EDTA on Epinephrine-induced Contraction.* In similar experiments, the disodium salt of ethylenediamine tetracetic acid (EDTA), in concentration equimolar to that of T_3, also potentiated the epinephrine-induced contraction under same conditions as described for T_3. Epinephrine was added to the bath 2-5 minutes after EDTA in these experiments.

4) In other experiments, a 1×10^{-5} concentration of epinephrine was incubated at 37°C in a 50 ml oxygenated Ringer's solution which contained 1 g/liter of glucose, a 4×10^{-7} concentration of copper acetate and a 2×10^{-6} concentration of T_3. A similar incubate without T_3 served as control. Aortic tissue was not present in these incubates. At intervals after beginning incubation, 0.5 ml aliquots from each incubate were injected intravenously into a pentobarbitalized cat and blood pressure changes recorded from carotid artery on smoked kymograph by a mercury manometer. Dose of epinephrine administered was

TABLE I. Effects on Epinephrine Contraction of Isolated Rabbit Aortic Strip.

Treatment	No. of rabbits	No. of strips	Avg % increase in: Contraction height	Avg % increase in: Duration of contraction
T_3 (1×10^{-6}), short epinephrine contraction—no added Cu^{++} in bath	1	2	54 (27–80)	73 (58–87)
T_3 (1×10^{-6}), long epinephrine contraction—no added Cu^{++} in bath	3	8	14 P >.05	
T_3 (1×10^{-6}), added Cu^{++} in bath	7	10	40 P <.01	61 P <.01
EDTA (5×10^{-7}),* added Cu^{++} in bath	5	8	31 P <.05	>200
EDTA (5×10^{-7}),* short epinephrine contraction—no added Cu^{++} in bath	1	3	32 (20–42)	>200
Ephedrine, no added Cu^{++} in bath	6	14	24 P <.05	
Ephedrine, added Cu^{++} in bath	3	7	10 P >.05	22 P >.05

* Concentration equimolar to that of L-T_3.

usually 2.5 μg/kg body weight.

As can be seen in representative recording in Fig. 1, pressor response to epinephrine from treated incubate was somewhat greater than that from control incubate after 15 and 30 minutes of incubation; at 60 minutes epinephrine from treated incubate produced a markedly greater pressor effect than the control; and at 90 minutes pressor response to control epinephrine had disappeared while there was still a definite pressor response to the treated epinephrine. It was determined in separate experiments that T_3 alone, in dose equivalent to that contained in incubate aliquots, produced no effect on cat blood pressure. Control incubates turned pink much sooner than those containing T_3. In similar experiments in which copper was *not* added to the incubates, there was no difference observed in pressor responses to epinephrine from either control or T_3-treated incubates. Moreover, there was no difference in times in which the incubates turned pink.

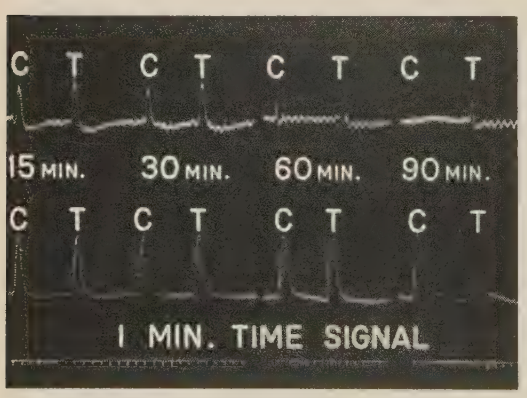

FIG. 1. Cat blood pressure response to epinephrine. Epinephrine incubated in Ringer's solution at 37°C with (T) and without T_3 (C). At intervals after beginning incubation, aliquots equivalent to 2.5 μg/kg epinephrine were taken from the incubates and inj. into a pentobarbitalized cat intrav. and arterial blood pressure recorded. Top recording shows results of representative experiment in which copper acetate was added to incubates and bottom recording shows results of representative experiment in which copper acetate was excluded from incubates.

5) *Effect of Ephedrine on Epinephrine-induced Contraction.* Experiments were designed to determine if amine oxidase inhibition was a possible factor in T_3-potentiation of epinephrine described above. After initial control injections of epinephrine, ephedrine was injected into the muscle bath to give a concentration of 1×10^{-6}. This concentration is one which has been previously reported to inhibit amine oxidase *in vitro* (4). Five to 10 minutes later, the response to epinephrine was again recorded. Ephedrine significantly potentiated the epinephrine contraction in absence of added copper in the bath, but only slightly or not at all when copper was present (Table I).

6) *Effect of T_3 on Barium Chloride-induced Contraction.* Concentration of barium chloride to evoke a contraction of the aortic strip was 4×10^{-4}. Height of contraction averaged about the same as that elicited by 1×10^{-7}

epinephrine, was well below maximum, and was similarly prolonged. T_3 (1×10^{-6}) failed to significantly potentiate barium chloride-induced contractions either in presence or absence of added copper in the bath.

Discussion. The results indicate that T_3 potentiates epinephrine-induced contraction of the isolated rabbit aortic strip in major part by a metal-dependent anti-oxidant effect. Moreover, this mechanism appears to be independent of aortic tissue. The evidence can be summarized as follows:

1. T_3 potentiated epinephrine when copper acetate was added to muscle bath but not in absence of copper. The potentiation in a few experiments in which copper was not added to the bath can probably be attributed to presence of trace metal contaminants in the bath. This is suggested by unusually short duration of epinephrine-induced contractions in these experiments.

2. EDTA, a potent chelating agent, potentiated the effect of epinephrine in similar experiments under same conditions.

3. T_3 delayed appearance of pink color in an incubate containing epinephrine, Ringer's solution and copper acetate. No delay was noted when copper was not added to T_3 treated incubate. The pink color is known to result from oxidation of epinephrine(5). Moreover, the pressor response of anesthetized cats to epinephrine incubated in presence of T_3 and copper (determined at intervals after beginning incubation) was evident long after the pressor response to "unprotected" epinephrine had disappeared. T_3 exerted no effect when copper was not added to the incubate.

The evidence thus indicates that T_3 delays metal-catalyzed oxidation of epinephrine. This explains in major part the potentiation of epinephrine-induced contraction of the isolated rabbit aortic strip. The mechanism of the anti-oxidant effect is probably mediated through the metal chelating action of T_3. Ability of T_3 to chelate copper and other heavy metals has recently been demonstrated by means of an acidometric method.† The strong metal complexing property of thyroxine has previously been indicated by Frieden(6) and Gemmill(7). An alternative explanation of the anti-oxidant effect of T_3 is suggested by more recent work of Frieden(8), who demonstrated that T_3 as well as thyroxine can reduce cupric ions to the cuprous form. This could also result in prevention of copper-catalyzed oxidation of epinephrine.

Amine oxidase inhibition does not appear to be a major factor contributing to T_3 potentiation of epinephrine. The evidence for this was that ephedrine, in concentration known to inhibit amine oxidase, produced effects which were the reverse of those exerted by T_3.

It may also be concluded that the potentiating effect of T_3 can not be attributed to T_3-induced general increase in sensitivity of rabbit aortic strip because T_3 failed to potentiate contraction induced by barium chloride.

Summary. L-3,3',5-Triiodothyronine (T_3) potentiated epinephrine-induced contractions of isolated rabbit aortic strip only under the following conditions: 1) copper acetate (2×10^{-7}) addition to the bath or 2) when trace metal contaminants were occasionally present in the bath, as suggested by short duration control responses to epinephrine. EDTA, a potent chelating agent, similarly potentiated epinephrine contractions. It was further demonstrated that T_3 delays metal-catalyzed oxidation of epinephrine in separate incubate experiments. This effect appears to be the major factor in potentiation of epinephrine contractions by T_3. It is further concluded that T_3 probably acts by virtue of its metal-chelating effect.

† Personal communication from Dr. Stuart P. Eriksen, Smith Kline & French Labs., Phila., Pa.

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Received September 8, 1959. P.S.E.B.M., 1960, v103.

Toxicity and Metabolism of 2-Cyanopropylamine in Rats, Chicks, and Turkeys.* (25490)

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Administration of beta aminopropionitrile (BAPN) to rats produces skeletal deformities and changes in walls of blood vessels resulting in internal hemorrhage and death(1-5). Similar toxic symptoms were produced in young turkeys(6) and in chicks(7) at lower levels of BAPN. Cyanoacetic acid was isolated from urine of rats fed BAPN(8). Our object was to study in turkeys, chicks, and rats the toxicity and metabolism of 2-cyanopropylamine (CPA), $NH_2CH_2CH(CH_3)CN$, a methyl analog of BAPN.

Methods and materials. 2-Cyanopropylamine was prepared by procedure of Dickey (9) in which concentrated aqueous ammonia was reacted with methacrylonitrile† in a rocking hydrogenation bomb for 2 hours at 135°C. The product was purified by distillation and obtained as colorless liquid, b.p. 75-76° (16 mm). Anal. Calcd. for $C_4H_8N_2$: N, 33.17. Found: N, 33.21. BAPN was used as neutral fumarate salt (2 BAPN · 1 fumaric acid).‡ 2-Cyanopropionic acid was synthesized by reacting beta-bromopropionic acid with sodium cyanide according to procedure of Hoffman and Barbier(10). *Animal diets and procedures.* The basal diet of Barnett *et al.*(6) was used in chick and turkey experiments, except that dried skimmilk replaced dried whey. Each group contained 10 birds, housed in heated batteries. Experimental diets and water were given *ad lib.* from first day after hatching. Birds were weighed at weekly intervals and severity of symptoms recorded by assigning a numerical score to leg deformities at each weighing. Gross autopsies§ were performed on animals which died during experiment. Day-old straight run, cross-bred (New Hampshire male X Single Comb White Leghorn female) chicks were used in Exp. 18. In Exp. 19 and 20 Beltsville Small White turkeys were used and the 2 experiments run for 5 weeks each. In the rat exp. 6 weanling male albino rats (Holtzman strain) were used. Rats had access to food for 8-9 hours each day and were kept in metabolism cages for collection of urine the rest of the time. Water was available at all times. Animals were fed a stock diet having the following composition in percent: ground yellow corn 28, ground wheat 28, powdered milk 11.2, soybean oil meal 9.3, linseed oil meal 9.3, alfalfa meal 7.5, butter 4.7, and NaCl 1.9. This diet has been used routinely as stock diet for rats in the Dept. of Biochemistry. Controlled feeding was continued for first 21 days of experiment after which food and water were given *ad lib.* for additional 14 days. At termination of experiment, 2 animals from each group were X-rayed.‖ All animals were then killed and gross autopsy observations made. Urine of rats from the 4 groups was collected under toluene for 21 days and stored in deep freeze cabinet until used. *Paper chromatography of rat urines.* Aliquots of approximately 20 μl of urine were spotted directly on Whatman No. 1 paper. The chromatograms were developed by an ascending technic with butanol-acetic acid-water (120:30:50)(11). After drying, amines and amino acids were detected by ninhydrin spray. Duplicate chromatograms were sprayed with 0.1% 2,6-dichlorophenolindophenol in alcohol(12) for detection of acidic components. *Isolation of cyanopropionic acid from rat urine.* Ninety ml of urine collected from rats on 0.48% CPA diet (group 3, Table III) were acidified with 2.5 ml of concentrated sulfuric acid and clarified by centrifuging. The clear acidified superna-

* Published with approval of Director of Wisconsin Agr. Exp. Station. Supported by Research Comm. of Graduate School from funds of Wisconsin Alumni Research Fn., Madison, and N.I.H., U.S.P.H.S.

† Generously supplied by Tennessee Eastman Co.

‡ Generously supplied by Abbott Labs.

§ We thank Dr. J. J. Lalich for autopsy results.

‖ We thank Dr. R. F. Douglas for X-ray pictures of the rats.

tant was extracted 6 times with 50 ml of ethyl acetate. Emulsions formed during extraction, were broken by centrifuging. The ethyl acetate extract was then washed 4 times in separatory funnel with small volumes of distilled water and the aqueous washings discarded. The clear ethyl acetate extract was evaporated at room temperature and the residue subjected to molecular distillation for 1 hour at 5 x 10^{-4} mm and 80-100°. The distillate was collected as an oily film on surface of condenser tube cooled with dry ice freezing mixture. Distilled material was washed from condenser with small volumes of ethyl acetate and the solution evaporated to about 2 ml. This concentrate was then examined by paper chromatography with butanol-acetic acid-water system(11) and with a solvent system composed of n-butanol saturated with 1.5 N ammonia(13). Presence of 2-cyanopropionic acid in the distillate was strongly indicated by appearance of paper chromatograms, and was confirmed by preparation of a crystalline cyclohexylamine salt. For this purpose the distillate was evaporated almost to dryness, the residue taken up in 2 to 3 ml of acetone and the acetone solution neutralized with cyclohexylamine. The mixture was cooled and the crystalline precipitate filtered off and recrystallized from acetone. Thirty mg of colorless needle crystals, m.p. 154-156.5 were obtained. Anal. Calcd. for $C_{10}H_{18}O_2N_2$: N, 14.13. Found: N, 14.36. For comparison, a sample of the same salt was prepared from synthetic DL-2-cyanopropionic acid and obtained as colorless needle crystals, m.p. 152-154.5. Anal. Calcd. for $C_{10}H_{18}O_2N_2$: C, 60.6; H, 9.15; N, 14.13. Found: C, 60.01; H, 8.68; N, 13.45. A mixed melting point of isolated and synthetic salts was 154-156°C. Infrared tracings on isolated and synthetic samples, taken in a potassium bromide pellet, were essentially indistinguishable.

TABLE I. Effects of BAPN Fumarate and 2-Cyanopropylamine (CPA) on Weight, Leg Deformity and Mortality of Chicks.

Additions to basal diet, %				
CPA	BAPN fumarate	Avg wt 4 wk, g	Leg score*	Mortality, %
.0	.0	282	5.	0
.0	.079	151	3.7	70
.036	"	85	1.	80
"	.0	288	5.	0
.072	.0	241	3.9	0
.108	.0	257	3.4	0

* Leg scores (avg of surviving birds): 1. Both legs severely bent; joints swollen. 2. One leg slightly, one severely bent; joints swollen. 3. Both legs slightly bent; joints swollen. 4. One leg normal; other slightly affected. 5. Normal.

Results. Animal experiments. Table I summarizes results of Exp. 18 on chicks. A level of 0.036% of CPA alone was not toxic but when mixed with 0.079% of BAPN fumarate, the mixture was appreciably more toxic than this level of BAPN alone. As percent of CPA was increased there was slight depression of growth rate, and severity of leg deformities increased. Table II shows results of Exp. 19 and 20. The symptoms produced by combination of CPA and BAPN were more severe in turkeys than in chicks, as all 10 poults died in the third week. As in Exp. 18, higher levels of CPA led to suppression of growth and increase in severity of leg and toe deformities. Exp. 20 (Table II) showed that 0.216% of CPA was at least as toxic as 0.079% of BAPN fumarate as judged by weight and by leg deformities. A level of 0.079% of BAPN fumarate is equivalent to 0.052% of CPA on a molar basis. It is clear that the "diluting effect" of added methyl group in CPA accounts for only a small part of the difference in toxicity between CPA and BAPN. Table III shows that growth and survival rate in rats were progressively reduced by increasing CPA levels. In all cases death resulted from aortic rupture. An additional toxic symptom observed in all animals on CPA was male priapism. Definite lathyritic deformities were not revealed by X-ray pictures of rats. Autopsy examination of surviving rats on 0.24% CPA revealed no significant difference from controls, although one rat died of aortic rupture during experiment. Of the 4 surviving animals on 0.48% CPA, one showed hemorrhage in area of aortic arch, normal aortae being present in the other three. Femurs, sterna, and vertebral columns were not obviously lathyritic.

Urinary metabolites. When the 4 unfractionated rat urines corresponding to groups shown in Table III were chromatographed on

TABLE II. Effects of BAPN Fumarate and 2-Cyanopropylamine (CPA) on Weight, Leg Deformity, Mortality and Massive Internal Hemorrhage in Beltsville Small White Turkeys.

Exp. No.	Additions to basal diet, % CPA	BAPN fumarate	Avg wt 5 wk, g	Leg score*	Mortality, %	Hemorrhage, % of original group
19	.0	.0	713	5.	0	0
	.0	.079	447	3.	90	90
	.036	"			100	30
	"	.0	651	4.7	0	0
	.072	.0	697	4.7	10	0
	.108	.0	587	3.9	10	0
20	.0	.0	510	5.	0	0
	.0	.079	433	3.	80	60
	.216	.0	316	1.	70	50

* Avg of surviving birds.

paper and the papers sprayed with ninhydrin, a purple zone with R_f approximately 0.56 was observed only in urines of Groups 2 and 3 and the zone was more pronounced in Group 3 than in Group 2. This zone was completely absent in both control and BAPN groups. Since known CPA gave an identical color with ninhydrin and showed the same R_f on paper chromatograms, it was concluded that unchanged CPA was present in above urines. Group 4 urine showed a green ninhydrin spot due to unchanged BAPN(8). Duplicate chromatograms of urines from Groups 2 and 3, when sprayed with diazotized sulfanilic acid (8), showed no orange spot characteristic of cyanoacetic acid.

If CPA were metabolized similar to BAPN (8), one would expect to find 2-cyanopropionic acid in the urine. The isolation procedure, patterned after that used for cyanoacetic acid (8), gave a concentrate which when examined on paper chromatograms displayed an acid zone at an R_f of about 0.77 in the butanol: acetic acid:water system and at 0.19 in the butanol - 1.5 N ammonia system. Identical R_f values were observed for synthetic 2-cyanopropionic acid in the corresponding solvent systems. Presence of this metabolite in urine was then confirmed by isolation of the crystalline cyclohexylamine salt which proved to be identical with an authentic sample. Metabolism of amino and alkyl nitriles was discussed by Merkow *et al.*(14). Following the same reasoning, one would expect that oxidation of the amino group of CPA to an aldehyde group would be catalysed by monoamine oxidase, and that the aldehyde group then would be oxidized to - COOH. No attempt was made to determine optical configuration of urinary 2-cyanopropionic acid or CPA, but this might be of interest as the CPA fed was a racemic mixture and its optical forms might be metabolized to unequal degrees.

TABLE III. Effect of 2-Cyanopropylamine (CPA) and BAPN Fumarate on Weight, Mortality, and Massive Internal Hemorrhage in Rats.

Group No.	Diets	Avg wt 5 wk, g	Mortality, %	Hemorrhage, % of original group
1	Basal only	162	0	0
2	Basal + .24% CPA	145	17	17
3	+ .48% "	101	33	33
4	+ .36% BAPN fumarate	127	50	50

Summary. Lathyrogenic activity of 2-cyanopropylamine (CPA), a methyl analog of BAPN, was studied in chicks, turkey poults and weanling rats. Dissecting aneurisms of the aortae resulted in all 3 species. Although characteristic deformities of leg bones appeared in chicks and turkeys, lathyritic osseous changes were not produced in rats. A toxic symptom produced in all rats by CPA was male priapism. A level of 0.216% CPA in the diet of turkey poults was at least as toxic as 0.079% of BAPN-fumarate. CPA was excreted in urine of rats as unchanged CPA and as 2-cyanopropionic acid.

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Received September 22, 1959. P.S.E.B.M., 1960, v103.

Influence of Changes in Serum Phosphorus and Creatinine on Respective Concentration in Pancreatic Juice. (25491)

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Few studies have been devoted to the relation between chemical composition of plasma and of secretions of the gastrointestinal tract. This report concerns the influence of changes in plasma content of inorganic phosphorus and creatinine upon their respective concentrations in pancreatic juice. Both elements can be elevated artificially to a high level in blood without danger for the laboratory animal. Ball(1) has shown that calcium, phosphorus and magnesium in pancreatic secretion are much lower than in serum (¼ to 1/20th). Normally, inorganic phosphorus concentration in pancreatic juice equals 1/20 of its plasma value. When plasma phosphorus concentration is raised to 6 times greater than normal, there is a 12 fold increase in concentration of this ion in the pancreatic secretion, this being 1/10 of plasma value.

Technic. Experiments were performed on dogs (20 kg) with duodenal fistula. A modification of the Thomas(2) method was used, by which a fine glass cannula may be introduced to collect pure pancreatic juice. In fasted dogs, amount of juice so collected is minimal; it increases when secretin is injected intravenously. To establish a constant rate of secretion, secretin (Boots) was injected at constant rate with syringe for continuous injection. Rate of pancreatic juice secretion was adjusted betwen 1 and 1.5 cc/min. This was considered sufficient to avoid errors due to pancreatic dead-space. Extreme limits for each individual pancreatic juice collection period were 0.54 cc and 2.6 cc/min. The first 10 cc of pancreatic juice were discarded. Each experiment consisted in measuring output and concentration of inorganic phosphorus (Exp. 1-4) or creatinine (Exp. 2-4) in pancreatic juice at various levels of plasma inorganic phosphorus or creatinine. In Exp. 1 and 2, phosphorus excretion was measured at normal level of serum phosphorus and at various degrees of increased phosphorus levels, in Exp. 3 and 4 only at various levels of hyperphosphoremia. Increased serum phosphorus was induced by injection of buffered solution of inorganic phosphates at first in concentrated, then in diluted form. Rate of injection was such as to obtain levels as constant as possible. In Exp. 2-4, creatinine was added to the liquid of perfusion. Each collection period lasted 4 to 10 minutes. Blood samples were taken at brief and regular intervals throughout experiment. After each induced change in blood level of phosphorus and creatinine, the new pancreatic collection was begun only after at least 5 cc of

TABLE IA. Effect of Increase of Plasma Inorganic Phosphorus on Ratio between Phosphorus Concentration in Pancreatic Juice and in Plasma of Dogs (4 Exp.).

Exp.	Phosphorus in plasma, mg/l	P. conc. in pancreatic juice / P. conc. in plasma	
1	49	.014	
	49	.010	
	49	.011	
	100	.015	
	108	.015	
	201	.021	
	276	.011	
	309	.012	
	337	.010	
	382	.008	
	385	.008	
		mean = .012	σ = .00381
2	53	.009	
	53	.008	
	117	.009	
	131	.010	
	207	.014	
	216	.009	
		mean = .010	σ = .00214
3	123	.008	
	128	.011	
	129	.009	
	205	.010	
	210	.011	
	213	.011	
	214	.011	
		mean = .010	σ = .00122
4	111	.007	
	113	.008	
	114	.007	
	111	.009	
	213	.008	
	218	.007	
	219	.006	
	219	.006	
	260	.007	
		mean = .007	σ = .001

TABLE IB. Effect of Increase of Plasma Creatinine on Ratio between Creatinine Concentration in Pancreatic Juice and in Plasma of Dogs (3 Exp.).

Exp.	Creatinine in plasma, mg/l	Creat. conc. in panc'tic juice / Creat. conc. in plasma	
2	7.7	(Creat. in pancreatic juice unmeasurable)	
	7.7		
	30.4	.17	
	36.4	.17	
	74.	.18	
	78.	.14	
		mean = .165	σ = .0173
3	50.2	.06	
	52.	.06	
	53.5	.06	
	108.	.08	
	115.	.08	
	119.5	.08	
	123.	.09	
		mean = .07	σ = .0129
4	54.	.06	
	55.5	.07	
	56.	.09	
	53.	.06	
	123.	.06	
	132.	.06	
	133.	.05	
	134.	.06	
	173.	.07	
		mean = .065	σ = .0113

pancreatic juice were obtained and discarded. After intravenous injection of radioactive phosphorus (P 32) radioactivity appears in the pancreatic secretion within 2 minutes or less. It may be concluded that the pancreatic "dead-space" is unimportant. To minimize error due to pancreatic "dead-space" in calculation of clearance of creatinine and inorganic phosphorus for each collection period, we divided the output of these substances by their plasma concentrations at mean time, less 3 minutes of each collection period. Inorganic phosphorus in plasma and pancreatic juice was measured by the technic of Berenblum and Chain(3); creatinine was measured by the method of Bonsnes and Taussky (4).

Results. I. *Effect of increased plasma inorganic phosphorus on its concentration in pancreatic juice* (Table IA). *a*—At a normal level of serum phosphorus, concentration of inorganic phosphorus in pancreatic juice is approximately 1% of its plasma concentration. These figures were obtained from measurements in 5 dogs. (In other experiments not included in Table I percentages up to 6% were found.)

b—Concentration of phosphorus in pancre-

atic juice is proportional to its concentration in plasma. The ratio between concentrations of inorganic phosphorus in plasma and pancreatic secretion remains constant even when there is 8-fold increase of plasma inorganic phosphorus (Exp. 1).

c—In another group of 3 experiments, parathyroid hormone was injected into the animal to study its effect on passage of phosphorus into the pancreatic secretion. After intravenous administration of 100 Collip units of Parathormone (Lilly), there was no change in amount of inorganic phosphorus passing into the pancreatic juice. These experiments lasted at least one hour after injection of parathormone.

II. *Effect of increased plasma creatinine on its concentration in pancreatic secretion* (Table IB). *a*. At a normal level of creatinine, amount of creatinine in pancreatic juice is too slight to be measured by the method used.

b. When level of creatinine in blood is increased, amount of creatinine in pancreatic secretion is much less than its plasma value. Ratio between concentrations in juice and plasma is respectively 0.165-0.07-0.065 in Exp. 2, 3 and 4.

c. As with phosphorus, there is a definite relationship between concentrations of creatinine in plasma and pancreatic juice. Their ratio remains constant whatever the creatinine content of plasma may be.

Comment. We were interested in the study of permeability of digestive glands to various substances normally present in plasma. Our investigation appears to be the only study of transfer of creatinine through the pancreas at various levels of serum creatinine. Other studies have been directed to permeability of this gland to urea and uric acid. These too, are found in much lower concentration in pancreatic juice than in plasma (Agren 5).

The relative lack of permeability for these products of protein metabolism seems to be a characteristic of the pancreas as compared to other digestive glands. The transfer of non-protein nitrogen, urea, uric acid and creatinine in saliva(6 to 12), stomach juice(7,10, 13 to 20), bile(5,7,13,18,21-24) and intestinal juice(7,25 to 27) has been investigated by various authors, each studying usually one aspect of the problem. There are some differences of opinion; however, concentrations of these compounds in plasma and in digestive secretions, except pancreas, are of the same order of magnitude. Uric acid seems to pass less readily in saliva(12) and especially in gastric juice(20) where it reaches, on the average, one-third of its plasma value. This difference is still much lower than that observed at pancreatic level for each derivative of protein metabolism, creatinine, urea, uric acid.

This relative lack of permeability of the pancreas to products of protein metabolism cannot be explained at present. More extensive research is needed concerning permeability of the digestive tract to ions and other plasma elements and the influence of changes in their plasma levels upon their concentrations in glandular secretion. How intestinal absorption of an element is affected by change in its concentration on one side of the intestinal barrier is a corollary problem, as yet insufficiently studied.

Summary. 1) Concentrations of inorganic phosphorus and creatinine in pancreatic secretion are much lower than their respective concentrations in plasma. 2) Any increase in inorganic phosphorus or creatinine content of plasma leads to proportional increase of their respective concentrations in the pancreatic secretion. 3) Ratios between concentrations of inorganic phosphorus in pancreatic secretion and in plasma remain constant, whatever the inorganic phosphorus content of plasma may be. The same is true for creatinine. 4) Relative lack of permeability for products of protein metabolism seems to be a characteristic of the pancreas, as compared to other digestive glands.

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Received October 6, 1959. P.S.E.B.M., 1960, v103.

Association of Resistance to Bile Salts and Virulence in *Salmonella typhimurium* Strains* (25492)

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A number of factors have been reported to influence virulence of various *Salmonella* species. Among these are endotoxins(1), Vi antigens(2), and resistance to action of complement(3). Certain nutritionally deficient strains have also been shown to be less virulent than those from which they were derived (4,5). Results of our study with *Salmonella typhimurium* indicate that in some cases ability to tolerate high concentrations of bile salts is accompanied by increased ability of the organism to persist in tissues of mice.

Methods. S. typhimurium strains used were differentiated by ability to form colonies on SS agar (Difco) which contains 0.85% Bacto Bile Salts No. 3 (Difco). Experiments in which individual components of this medium were tested showed that bile salts were responsible for the inhibitory effect. A brief description of the *S. typhimurium* strains follows: RIA, a relatively avirulent strain unable to form colonies on SS agar, obtained from H. A. Schneider, Rockefeller Inst. for Med. Research; RIA/SS_1, RIA/SS_2, mutants of RIA able to grow on SS agar, isolated by selection on bile agar made by adding 1.5% agar to liquid bile medium described under *in vitro* tests; RIA/SS_3, mutant of RIA able to grow on SS agar, isolated from a mouse inoculated with 4 x 10^6 cells of parent strain; *rev*, SS sensitive reversion of RIA/SS_1, unable to grow on SS agar. Bacteria for use in mouse virulence tests were grown overnight in penassay broth (Difco) at 37°C with aeration and diluted in saline. Assays for viable cell count were made on nutrient agar with control platings on SS agar. Mice were inoculated intraperitoneally with 0.2 ml of various dilutions. Animals employed in all virulence tests were from a colony of *Salmonella* free white Swiss mice maintained in this laboratory. They were originally obtained from Ft. Detrick, Frederick, Md. Equal numbers of males and females 5 to 8 weeks old were used in each experiment. Animals which died were examined for necrotic foci on liver, and spleen

* This investigation supported by contract with U. S. A. Chemical Corps, Fort Detrick, Md.

TABLE I. Comparison of Virulence of *Salmonella typhimurium* Strains Inoculated Intraperitoneally into White Swiss Mice.

Strain	Cells in inoculum* $\times 10^4$	No. survivors/No. inoculated	+ isolations from spleens†	No. with necrotic foci on livers†
RIA	5	44/52	10	5
SS_1	6	43/64	41	21
SS_2	5	10/18	10	6
SS_3	8	12/20	11	9
rev	7	29/34	8	0

* Avg for all experiments included in Table.
† Figures given are for surviving mice sacrificed 28 days after inoculation.

tissue was cultured on nutrient and SS agar to determine whether salmonellae were present. Surviving mice were sacrificed at 28 days and subjected to similar examination. In some experiments measuremnts for spleen size were made on animals that survived 5 days or longer after inoculations. Prior to this time pathological changes were seldom observed although bacteria were invariably present in spleen tissue. *In vitro tests.* A liquid medium essentially like SS agar was used to determine the effect of bile salts on resistant and sensitive strains of bacteria. Its composition was as follows: proteose-peptone (Difco), 10 g; lactose, 10 g; Bacto Bile Salts No. 3, 8.5 g; sodium citrate, 8.5 g; sodium thiosulfate, 8.5 g; distilled water, 1000 ml. 0.5% sodium desoxycholate could be substituted for bile salts. Known numbers of cells from overnight aerated penassay broth cultures of strains RIA and RIA/SS_1 were added to tubes containing 10 ml of liquid bile medium and incubated with aeration at 37°C. Samples of each culture were removed at intervals up to 24 hours and assayed for viable count on nutrient agar.

Results. Virulence tests. Data from several experiments have been pooled in Table I. The enhanced virulence of bile resistant organisms was evidenced less by ability to initiate fatal infection than by prolonged persistence within the host and alteration of internal organs. Although some overlapping occurred, 3 quite distinct classes resulted when spleen measurements were tabulated. Spleens from mice receiving sterile saline only were nearly all less than 19 mm in length. From those inoculated with strain RIA, lengths to 23 mm were obtained, while in animals which received injections of bile resistant bacteria, most spleens exceeded 23 mm. In some instances adhesions, as well as extensive areas of necrotic tissue, were noted in livers of mice inoculated with bile resistant organisms, while in mice inoculated with sensitive organisms no adhesions were observed.

In vitro tests. Results obtained when strains RIA and RIA/SS_1 were inoculated into liquid bile medium (Fig. 1) indicate that the sensitive strain rapidly loses viability under these conditions. Cells of the resistant strain appeared normal when examined microscopically while the sensitive culture contained many small, spherical bodies and remnants of lysed cells. Motility had almost completely ceased after 4 hours. Loss in viability of sensitive cells could be lessened by diluting samples into peptone broth plus 20% sucrose and

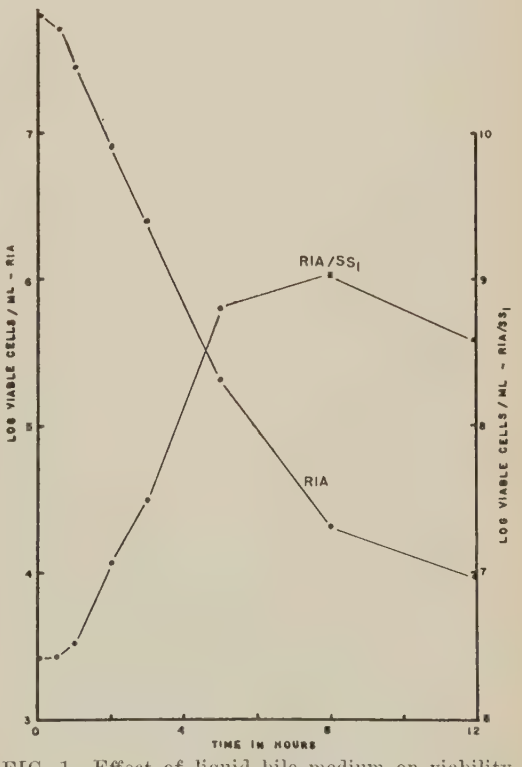

FIG. 1. Effect of liquid bile medium on viability of *Salmonella typhimurium* strains RIA and RIA/SS_1. Final counts, made at 24 hr, were as follows: RIA, 3×10^1/ml; RIA/SS_1, 2×10^8/ml.

incubating for a short time prior to plating on nutrient agar. Since the protective effect was absent when cells were diluted in peptone broth alone, presumably the high osmotic pressure of the sucrose solution was preventing lysis and allowing the cells to return to normal form(6).

Summary and conclusions. A strain of *Salmonella typhimurium,* RIA, unable to grow in the presence of high concentrations of bile salts was less virulent for mice than 3 mutants which were not inhibited under the same conditions. Two of these were isolated on inhibitory medium, the third independently from a mouse which died following infection with avirulent strain RIA. A sensitive reversion from one of the resistant mutants exhibited a virulence pattern similar to that of the sensitive parent strain. Resistant bacteria produced greater damage to internal organs and were able to persist for longer periods of time in host animals although the incidence of fatal infection was only somewhat higher than in mice infected with bile sensitive organisms. Limited studies comparing bile sensitive and resistant strains of *Salmonella paratyphi* B yielded similar but less striking results(7). These observations indicated that salmonellae able to withstand the effect of bile salts were also better able to establish chronic infection in mice leaving relatively large numbers of survivors as carriers of the organisms.

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Received October 7, 1959. P.S.E.B.M., 1960, v103.

Estrogen Antagonisms: Effects of Gluco- and Mineralocorticoids on Estrone-Induced Uterine Growth.* (25493)

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The actions of estrogenic hormones are antagonized by a variety of other steroidal substances(1). We have attempted to compare the effects of various types of steroids as antagonists of estrogens. The effects of representatives of each of the 5 major classes of steroid hormones (estrogens, androgens, progestins, mineralo-, and glucocorticoids(2)), were studied in each available estrogen test. Recently we discussed the effects of progesterone, testosterone propionate and 17-ethyl-19-nortestosterone on uterine growth produced in mice by moderate dose of estrone(3). Similar studies also examined quantitative relationships among a long series of relatives of 19-nortestosterone(4). The present communication extends these observations to the effects of adrenal cortical hormones in our standard mouse uterine growth assay.

Materials and methods. The methods employed have been described(3,4,5,6). Briefly, test compound was mixed in solution with 0.3 μg of estrone; total doses were contained in 0.3 ml of corn oil. Mice received 0.1 ml of solution daily for 3 days, starting at 23 days of age, and were sacrificed on the 4th. Mean uterine wet weights from groups of 8-10 mice were used for analysis. The only deviations from these methods were in the separate administration of gluco-corticoids as microcrystalline suspensions and of the aldosterone as an alcohol-water solution.

Results and discussion. The glucocorticoids,

*We are indebted to Dr. R. T. Hill, Dept. of H.E.W., and to Ciba Corp. for 2 mg of aldosterone 21-mono-acetate. Dr. C. M. Kagawa of G. D. Searle and Co. assayed this material in his sodium-retaining test.

TABLE I. Effects of Cortisol Acetate on Estrone-Induced Uterine Growth in Mice.

Dose (μg)			
Estrone	Cortisol	N	Mean uterine wt (mg)
.3	0	10	30.0
.3	100	9	32.8
.3	"	10	28.5
.3	300	10	33.6
.3	"	10	35.4
.3	1000	10	29.2

Estimated slope of fitted dose-response curve does not differ from zero: b = .32 ± 4.09.

cortisone acetate and cortisol acetate, appeared to have little effect upon uterine growth produced by 0.3 μg of estrone. Cortisol acetate was examined in a single test (Table I); at doses up to 1 mg it had no marked inhibitory effect upon the estrone-stimulated uterus. Cortisone acetate was studied in tests over a broad range of doses (Fig. 1). Although inspection of data suggested no interaction, a significance test seemed appropriate. Dose-response distribution was divided into 4 quadrats by a horizontal line through the mean response of 5 concurrent estrone-treated groups, and by a vertical line through the median dose. The number of points falling in each quadrat was determined and randomness of distribution was confirmed by a 4-fold contingency test ($\chi^2 = 0.287$, df = 1, $P > 0.59$). Thus cortisone acetate appeared to have no significant effect upon estrone-induced uterine growth.

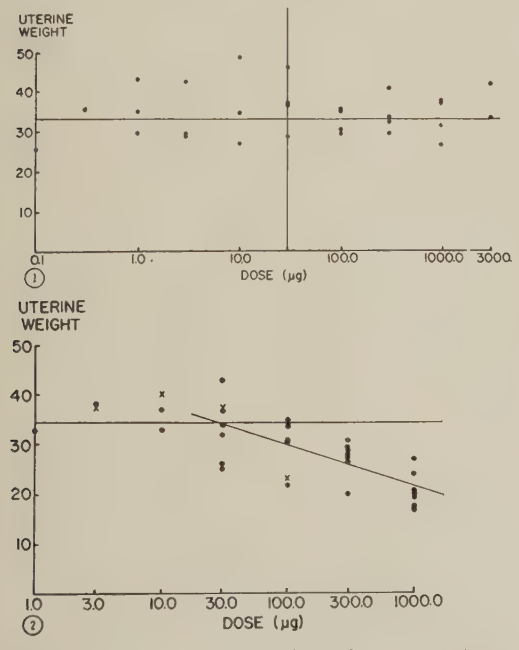

FIG. 1. Lack of effect of cortisone acetate on uterine response to .3 μg of estrone. Each point represents mean of 8-10 mice.

FIG. 2. Effects of desoxycorticosterone (●) and aldosterone (×) on uterine response to 0.3 μg of estrone. Aldosterone point at 100 μg is mean of 5 mice; all other points are mean of 8-10 animals. Fitted line for DCA: Uterine wt = 46.5 − 8.21 log dose. b = 8.21 ± 1.14.

In contradistinction to these findings Szego and Roberts(7) and Szego(8) showed that injected corticoids and stress counteracted water imbibition effects of estradiol-17β. Velardo(9) also found that glucocorticoids inhibited uterine growth produced by estradiol-17β. Rats were employed in each of these studies. Perhaps species differences are a partial explanation for contrasting results. Another difference from our procedure was the use of estradiol-17β as standard uterine growth stimulator.

Desoxycorticosterone also inhibited uterine water imbibition stimulated by estradiol-17β in rats(7), although Szego(8) pointed out later that results with this material were not consistent. Velardo(9) showed inhibition of estradiol-induced rat uterine growth with DCA. In our hands DCA was an inhibitor of estrone-stimulated uterine growth in intact, immature mice (Fig. 2). In comparison with our previous studies with relatives of 19-nortestosterone(4), DCA appears to be a weak estrone antagonist, only about 10% as active as progesterone. Qualitatively, there can be no doubt of the reality of this inhibition since the negative slope of dose response curve was highly significant ($t = 5.006$, df = 25, $P < 0.01$).

In addition to desoxycorticosterone, we had a small sample of aldosterone available for preliminary testing. The few data available for aldosterone suggest that this material is also an estrone antagonist, and that its potency is not remarkably different from that for DCA (Fig. 2). Since this particular sample of aldosterone assayed at about 50 times more potent than DCA in a rat salt-retaining test, it appears that little correlation exists between these 2 activities.

Summary. Uterine growth in intact, immature mice was stimulated by injection of 0.3

μg of estrone. When administered simultaneously, neither cortisone acetate nor cortisol acetate had any evident inhibitory effect. DCA, on the other hand, was a definite but weak estrone antagonist (10% as potent as progesterone). Aldosterone was active; its potency appeared to be about 10% progesterone.

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Received October 13, 1959. P.S.E.B.M., 1960, v103.

Lack of Relationship between Body Weight and Pharmacological Effect Exemplified by Histamine Toxicity in Mice.* (25494)

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The pharmacologic effects of many drugs and toxins appear to be proportional to their dose/unit body weight; for some drugs this may not be the case. The following study shows that toxicity of histamine in mice is not directly related to body weight.

Methods. Albino mice of Swiss strain weighing 15 to 25 g were used. Each group tested consisted of mice with a mean weight within $\pm$ 1 g of the desired mean body weight and with no individual mouse deviating by more than 1 g from the mean. Histamine diphosphate was dissolved in appropriate concentrations in 0.9% NaCl so that each animal received the desired dose in 0.2 ml of solution. Within each weight group all animals received the same dose of histamine diphosphate regardless of exact body weight of each animal. All injections were made intraperitoneally and mortality was recorded over a 24 hour period even though all animals that succumbed terminated within 6 hours after injection.

Results. A total of 360 mice were used in 18 groups of 20 mice each. The LD_{50} of histamine injected intraperitoneally was first determined in mice weighing 18 $\pm$ 1 g. Seven dose levels were utilized. Results and their statistical analysis are shown in Table I. Doses of 1.6 to 2.5 mg/g body weight produced submaximal responses. This dose range was then used to reevaluate the LD_{50} in 2 groups of mice, one of 18 $\pm$ 1 g and another of 25 $\pm$ 1 g. Both groups were tested simultaneously at 3 identical dose levels on a mg/g basis. Results are tabulated in Table II. When the doses are taken on a mg/g basis the smaller mice (18 $\pm$ 1 g) seem to have a larger LD_{50} than larger mice (25 $\pm$ 1 g). This difference is statistically significant and could have been interpreted as indicating that smaller animals are more resistant to histamine than larger. However the ratio of the two LD_{50}'s, *i.e.,* 1.29 with confidence limits of 1.15 to 1.44, is very close to the inverse ratio of weights of the 2 groups 25/18 = 1.39 suggesting that the difference between the 2 groups may be entirely due to the assignment of doses on body weight basis. In fact when the doses are expressed as mg/mouse, the results of the 2 groups fit a single dose-response probit regression indicating that they correpond to a single homogeneous population with an LD_{50} of 40 mg/mouse. This is further substantiated from the results of 3 different

* Supported by grant from Parke, Davis and Co., Detroit, Mich.

TABLE I. Histamine Toxicity Mice Weighing 18 ± 1 g* (20 Animals/Dose Group).

	Histamine diphosphate (mg/g body wt)	(mg/mouse)	Mortality (%)
	1.5	28	0
	1.7	31	20
	1.9	34	30
	2.1	38	35
	2.3	41	45
	2.5	45	65
	2.7	49	85
LD_{50}	2.3	41	
95% conf. limits	2.2 to 2.4	40 to 43	

* Within each dose group all animals were inj. with the same dose of histamine diphosphate intraper. Mortality was recorded over 24 hr after inj.

weight groups given the same dose on a mg/g basis (Table III A). As expected, mortality was higher in the larger animals than in smaller mice given the same dose on unit weight basis. When the doses are translated in mg/mouse the same data (Table III A) form a single regression with an LD_{50} of 36 mg/mouse (95% confidence limits 32 to 40). By contrast mortalities are very similar when 3 groups injected with the same dose on a mg/mouse basis are compared (Table III B). By translating the mortalities into probits the variance of the results under A and B (Table III) can be compared. The variance of the results is about 9.5 times larger when doses are expressed on a mg/g basis as compared to the results when doses are given on a mg/mouse basis (Tables III A *vs.* III B).

TABLE II. Histamine Toxicity in Mice Weighing 18 ± 1 and 25 ± 1 g (20 Animals/Dose Group).

Mouse wt (g)	Histamine diphosphate (mg/g)	(mg/mouse)	Mortality (%)
18 ± 1	1.6	29	20
"	2.0	36	30
"	2.4	43	60
LD_{50}	2.2	40	
95% conf. limits	2.0 to 2.5	36 to 45	
25 ± 1	1.6	40	30
"	2.0	50	80
"	2.4	60	90
LD_{50}	1.7	43	
95% conf. limits	1.5 to 1.9	38 to 48	
Potency ratio 18 *vs* 25 g	1.29	.93	
95% conf. limits	1.15 to 1.44	.85 to 1.02	

Both groups combined on basis of mg/mouse give an LD_{50} (and 95% confidence limits) of 40 (36 to 44).

Discussion. It is apparent from the results and statistical analyses that histamine toxicity in mice is represented more generally by a regression relating dose per animal rather than dose per unit body weight. This seems to be applicable in the range of body weights tested (15 to 25 g).

When doses are expressed in terms of unit body weight (mg/g) histamine appears to be less toxic in smaller than in larger animals. This is clearly an artifact as it is entirely dependent upon the assumption that the pharmacological effect of a given dose is a function of body weight. This assumption can not be justified by the data. The simplest explanation of the available data is that histamine toxicity in mice is an example of a pharmacological action where the dose producing a given effect is not a function of body weight, at least in the weight ranges tested.

TABLE III. Histamine Toxicity in Mice of 3 Different Weight Groups (20 Animals/Group).

Mouse wt (g)	Histamine diphosphate (mg/g)	(mg/mouse)	Mortality (%)	(probits)
		A		
15 ± 1	2.0	30	30	4.47
20 ± 1	2.0	40	60	5.26
25 ± 1	2.0	50	85	6.04
		B		
15 ± 1	2.67	40	50	5.00
20 ± 1	2.0	40	60	5.26
25 ± 1	1.6	40	40	4.75

Probit variance for mortalities of groups under A is 0.616 and that of those under B is 0.065 giving a variance ratio of 9.48.

It may be suggested that other variables inherent in estimation of histamine toxicity in mice may be so large as to mask any contribution produced by differences in body weight. This suggestion is not tenable in this case because correction for body weight actually increases variation to the extent that differences in toxicities between different weight groups become statistically significant. Alternatively it may be held that protective mechanisms (such as rate of inactivation and excretion) may bear the same relationship to body weight as those factors which contribute to toxicity

(such as rate of absorption and total body water) and thus the contribution of body weight is cancelled. In any event, the applicability of the results reported here may not be limited to this particular experimental model since it has been concluded(1) that for several pharmacological agents the lethal dose for small animals is usually a little larger than for large animals when both are expressed on a unit weight basis.

The above considerations are not intended to imply that the same relationship exists with other drugs or species or even with the same drug when a different pharmacological effect is evaluated. However they do emphasize that dependence of the dose on body weight can not be implicitly assumed in the absence of data showing that a relationship actually exists. Such a dependence may be practically zero, as in the present case, or it may be represented by any fraction of unity. In the later case it is best established by covariance analysis, which has been reported(2). It is noteworthy that a similar lack of a relation between body weight and toxicity has been shown to exist in the case of the lethal effects of botulinal toxins on mice(3) and alpha-naphthyl-thiourea in rats(4).

Summary. Data from 360 mice are presented which show that the toxicity of intraperitoneal histamine is independent of body weight. Adjustment for body weight in the range of 15 to 25 g increased the variation so that smaller animals appeared to be less sensitive to histamine than larger mice. Possible mechanisms are discussed.

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Received October 19, 1959. P.S.E.B.M., 1960, v103.

Initiation of Lactation in Rats with Hypothalamic or Cerebral Tissue.*† (25495)

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Recently we demonstrated that epinephrine, acetylcholine, serotonin and reserpine can initiate lactation in estrogen-primed virgin rats, and maintain mammary secretion in postpartum rats after litter removal(1,2). Numerous other drugs and a variety of stressful stimuli can also initiate mammary secretion in rats (unpublished). These drugs and stressful stimuli are believed to induce release of prolactin and probably ACTH from the anterior hypophysis, since administration of prolactin alone is ineffective in initiating lactation in intact rats and requires the addition of ACTH or glucocorticoids(3). Large doses of ACTH or glucocorticoids alone may induce mammary secretion in intact rats, presumably by stimulating pituitary prolactin release(4). Harris(5) and others have suggested that the hypothalamus and perhaps other portions of the brain produce neurohormones which reach the anterior pituitary *via* the hypophyseal portal vessels and induce release of its hormones. Thus hypothalamic extracts have been shown to elicit ACTH discharge(6,7) and pineal extracts to evoke aldosterone release(8). It was of interest, therefore, to determine whether hypothalamic or cerebral tissue from rats could induce release of sufficient prolactin and probably ACTH to initiate lactation in rats.

Methods. Brain tissue was taken from postpartum or estrogen-treated mature female rats (Carworth strain). They were killed with

* Published with approval of Mich. Agr. Exp. Sta. as Jour. Art. No. 2516.

† This work was supported in part by NIH grant.

TABLE I. Effects of Different Rat Tissues on Initiation of Mammary Secretion in Rats.

Treatment (5 rats/group)			No. rats with secretion
Tissue containing hypothalamus	29.4 mg,	½ twice daily	4
	29.4	1 × daily	4
	65.6	*Idem*	4
Cerebrum	29.4	"	2
	64.7	"	1
	217.6	"	1
Kidney	64.0	"	0
Liver	64.0	"	0
.85% saline		"	0

ether anesthesia and the whole brain was carefully lifted from the skull at the same time the connection to the pituitary stalk was severed. The tissue between the optic chiasma and mammillary bodies containing the hypothalamus was carefully excised, weighed, dropped into a vial and immediately placed in a freezer at a temperature of −15°C. When a sufficient number of such tissues were collected, they were macerated with a small mortar and pestle and suspended in .85% saline. Average wet weight of this tissue/rat was about 25 mg. Equivalent amounts of rat cerebrum, kidney or liver were similarly prepared for injection. A control group of 5 rats was injected subcutaneously with .85% saline daily. The tissues were injected subcutaneously in .2 cc volumes daily into groups of 5 mature rats each for 5 days after the rats had been injected subcutaneously for 10 days with 10 μg estradiol daily to develop their mammary glands. On the 6th day the rats were killed and their right inguinal mammary glands were removed for histological examination. To check on possible prolactin contamination, 29.2 mg of hypothalamic tissue was injected intradermally over one crop sac of each of 5 mature White Carneau pigeons for 5 days and saline was injected over the other crop sac. On 6th day the pigeons were killed, and the crop sacs were removed and examined macroscopically for evidence of stimulation.

Results. The amount of tissue injected/rat and their lactational responses are shown in Table I. It can be seen that tissue containing the hypothalamus induced mammary secretion in 4 out of 5 rats in each of the 3 groups injected, irrespective of the dose or schedule of administration. Mammary maintenance was also noted in the 3 rats not showing secretion, indicating at least a partial response to the tissue containing the hypothalamus. In the 3 groups given cerebral tissue, 2 out of 5 rats showed lactational responses to the lowest dose and 1 out of 5 rats in each of the 2 groups given the higher doses of this tissue. No mammary secretion was observed in rats when liver, kidney or saline was injected, and the 5 pigeons given suspensions of tissues containing the hypothalamus did not show a crop gland response. Representative histological sections of mammary glands from saline and hypothalamic-injected rats are shown in Fig. 1-2.

Discussion. These results show that rat hypothalamus and possibly cerebral tissue, can initiate mammary secretion in estrogen-primed rats. The tissue containing the hypothalamus induced lactation in 12 out of 15 rats and at least maintained the mammary structure in the other 3 rats, whereas cerebral tissue elicited mammary responses in only 4 out of 15 rats. Inability of the tissue containing the hypothalamus to elicit pigeon crop milk secretion by the sensitive intradermal test indicates that the active principle is not prolactin. Presumably this tissue induced discharge of prolactin and probably ACTH from the anterior pituitary. It is not clear why the higher doses of cerebral tissue failed to elicit more responses than the lower doses, although its effects may not be as specific as those of the hypothalamus. Failure of liver or kidney tissue to induce mammary secretion suggests that in amounts equivalent to the 2

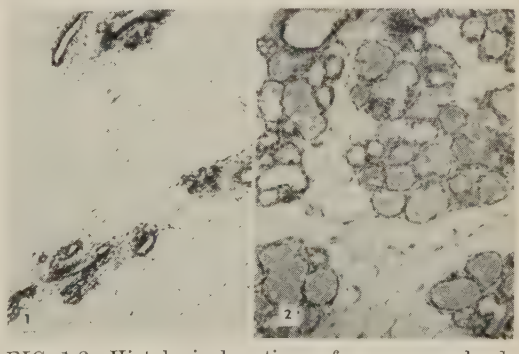

FIG. 1-2. Histological sections of mammary glands from rats inj. with (1) saline (2) tissue containing hypothalamus. ×63.

brain tissues, they do not exhibit prolactin and ACTH releasing ability possessed by the brain tissues. It is not known whether these brain tissues acted directly on the anterior hypophysis or indirectly through other centers. Preliminary assays of bovine hypothalamic and cerebral cortical tissues‡ show that these may also induce mammary secretion in rats.

Summary. Rat tissue containing the hypothalamus, cerebral tissue, kidney or liver were injected subcutaneously in saline suspension into rats for 5 days, after they had previously been injected with estradiol for 10 days to develop their mammary glands. Saline alone was injected into control rats. The tissue containing the hypothalamus initiated lactation in 12 out of 15 rats; cerebrum in 4 out of 15 rats; and liver, kidney or saline in none out of 5 rats each. Tissue containing the hypothalamus was injected intradermally over crop sacs of 5 pigeons for 5 days and failed to induce a response, indicating that the active principle is not prolactin. It is suggested that rat hypothalamus and possibly cerebral tissue produce release of prolactin and probably ACTH from the anterior pituitary in amounts sufficient to initiate lactation.

‡ Actone-dried preparations of these tissues were kindly supplied by Dr. C. E. Graham, Wilson Labs., Chicago.

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Received October 19, 1959. P.S.E.B.M., 1960, v103.

Pituitary ACTH Levels During Adrenal Involution Following Thiouracil.* (25496)

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Adrenal atrophy following administration of goitrogen thiouracil has been amply demonstrated(1,2,3,4) but the causal mechanism has not been fully elucidated. Atrophy of the adrenal cortex after thiouracil and inability to obtain adrenal hypertrophy following exposure to cold in thiouracil fed rats led Zarrow and Money(5) to conclude that adrenal involution was due either to decreased ACTH production by the pituitary or decreased sensitivity of the adrenal to ACTH. Subsequent studies by Zarrow and Zarrow(6) showing that the atrophic adrenal gland of thiouracil treated rat retains its sensitivity to ACTH were interpreted to indicate that adrenal involution in thiouracil fed rats is caused by decreased ACTH secretion. In the present experiment levels of pituitary ACTH were measured in rats showing adrenal involution induced by feeding thiouracil.

Materials and methods. Normal male albino rats, descendants from Sprague-Dawley strain weighing 150 ± 10 g were separated into 2 groups. Ten rats were kept as untreated controls and were fed Rockland rat

TABLE I. Mean Body and Fresh Organ Weights of Male Rats Fed a Diet Containing 0.3% Thiouracil for 3 Weeks.

Group	Body wt, g	Fresh organ wt, mg/100 g body wt		
		Thyroid	Adrenals	Pituitary
Thiouracil	196 ± 6*	44.7 ±2.8	13.1 ± .2	4.6 ±.3
Controls	246 ±17	7.2 ± .7	18.3 ±1.2	2.8 ±.3

* Stand. error of mean. All differences significant at P .05.

* The author gratefully acknowledges technical assistance of William Watson and Joan Michalski.

TABLE II. Mean Pituitary Weights and ACTH Content of Male Rats Fed a Diet Containing 0.3% Thiouracil for 3 Weeks.

	Pituitary wt				Pituitary ACTH		
	mg		mg/100 g body wt		U.S.P. milliunits		
Group	Fresh	Dry*	Fresh	Dry	Per mg fresh	Per mg dry	Per pituitary
Thiouracil	9	1.6	4.6	.8	10.5	59	94
Controls	7	1.4	2.8	.6	39.4	187	262

* Acetone-dried.

diet and tap water *ad lib*. Six other rats were fed Rockland rat diet containing 0.3% thiouracil (Schwarz Labs) and tap water *ad lib*. All animals were kept in quarters with controlled temperature (78 ± 2°F) and humidity (60 ± 5%). At the end of 3 week experimental period, animals were weighed and sacrificed with ether. Immediately thereafter, their pituitary glands were removed, weighed on torsion balance, and quickly placed in cold acetone. Thyroids and adrenals were also weighed at autopsy. After 24 hours in acetone pituitary glands were air dried at room temperature, reweighed and pulverized. For ACTH assay, the pituitary powder from thiouracil or control rats was suspended in 0.9% saline and assayed by intravenous U.S. Pharmacopeia (USP) ACTH assay against USP Corticotropin Reference Standard.

Results. Mean body and fresh organ weights after 3 week experimental period are shown in Table I. Pituitary weights and ACTH contents are summarized in Table II.

Thiouracil rats showed an increase in fresh thyroid and pituitary weights over untreated controls, but no significant difference in acetone-dried pituitary weights was evident. This indicates that the increase in fresh pituitary weight of thiouracil rats was largely due to higher water and/or fat content.

Body weights, adrenal weights and pituitary ACTH levels were lower in the thiouracil rats. Pituitary ACTH content of thiouracil rats was one-third or less than that of controls whether expressed in terms of units/mg of fresh weight, dry weight, or total units/pituitary gland.

Discussion. Adrenal-thyroid interaction was first suggested by Hoskins(7) when he reported adrenal hypertrophy after treatment with thyroid extract. Since then, adrenal atrophy following thyroidectomy(4,8) or treatment with thiouracil(1-4,9) has been shown in various species, but the mechanism of interaction has remained obscure.

When the hypothyroid state is induced by surgical thyroidectomy or thiouracil (a form of chemical thyroidectomy), the following hypotheses may be considered as far as adrenocortical atrophy and hypofunction(9,10): 1) A metabolic effect on adrenals due to lack of thyroxine, 2) A decrease in ACTH secretion by direct effect or by shift in pituitary production from ACTH to TSH. Since adrenal atrophy does occur following surgical thyroidectomy in absence of thiouracil and the condition can be corrected by small simultaneous doses of thyroxine(4), a direct toxic effect by goitrogen is unlikely.

The present findings of decreased pituitary ACTH levels following thiouracil strongly support the hypothesis first suggested by Zarrow and Zarrow(6), namely, that adrenal atrophy of the thiouracil rat is caused by decreased ACTH secretion. It may be, of course, that low levels of both thyroxine and ACTH are responsible for the adrenal involution effect, since lack of circulating thyroxine could quite conceivably aggravate the effect of low ACTH secretion.

Conclusions. Feeding rats a diet containing 0.3% thiouracil for 3 weeks brought about adrenal atrophy and thyroid and pituitary enlargement as compared to non-thiouracil controls. Pituitary ACTH content of thiouracil rats was less than ⅓ that in controls. The data support the hypothesis that adrenal atrophy following thiouracil is caused by lowered ACTH titers.

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Received October 21, 1959. P.S.E.B.M., 1960, v103.

Effect of N-(1-Methyl-2,3,di-p-chlorophenylpropyl)-Maleamic Acid (Benzmalecene) on Serum Lipids and Lipoproteins.* (25497)

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Among hypocholesterolemic agents recently made available for clinical trial is N-(1-methyl - 2,3,di-p-chlorophenylpropyl) - maleamic acid, Benzmalecene,† said to inhibit incorporation of 1-C^{14} acetate and 2-C^{14} mevalonic acid into cholesterol by rat liver homogenates, to inhibit development of hypercholesterolemia in chickens fed a diet containing 2% cholesterol and 5% cottonseed oil, to reduce the magnitude of cholesterolemia in rats fed a natural diet to which saturated fat was added, and to reduce serum cholesterol levels in dogs maintained on standard laboratory rations(1). We administered Benzmalecene to one hypercholesterolemic human subject and 2 normocholesterolemic subjects and noted hypocholesterolemic effects in each. Unusual changes in serum lipid pattern were induced.

Methods. Serum total and unesterified cholesterol and phospholipid concentrations and cholesterol and phospholipid content of serum lipoprotein fractions obtained by differential preparative ultracentrifugation were determined by methods we previously reported(2). "Phospholipids" were calculated by multiplying lipid P values by 25. Serum triglycerides were determined by the method of Van Handel and Zilversmit(3). Benzmalecene was administered to the following subjects: (1) PB a 39 year old woman with familial hypercholesterolemia who received 1 g/day for 7 days followed by 1.5 g/day for next 7 days; (2) ABB a 41 year old man with Klinefelter's syndrome who received 1.5 g/day for 10 days; (3) GM a 27 year old man with myotonic dystrophy and hypogonadism who received 1.5 g/day for 12 days. Both pre- and post-treatment control sera were studied. Post-treatment sera were obtained beginning 12 to 21 days after cessation of treatment.

Results. No change in serum transaminase (glutamic-oxaloacetic and glutamic-pyruvic) or van den Bergh levels were noted during the relatively short period of study. (Bromsulphalein retention has been noted in chronic toxicity studies in animals(1)). The compound uniformly produced anorexia or nausea which prompted one subject to refuse further medication after 12 days of treatment. No dietary restrictions were imposed and no changes in weight were noted. Since weight loss did not occur, the changes in serum lipids are not attributable to reduced caloric intake. The influence of anorexia *per se* on serum lipids is unknown.

The effects on serum lipids were similar in all subjects (Table I). The decrease in serum total cholesterol was limited to esterified cholesterol. Analysis of the ultracentrifugally separated lipoprotein fractions of densities <1.063 g/ml (includes "β" and all lower

* This study supported by grants from Nat. Heart Inst., U.S.P.H.S. and Oklahoma State Heart Assn.

† Merck, Sharp and Dohme Research Labs., West Point, Pa.

TABLE I. Serum Lipid Values, mg %.

Subject	No. of samples	Rx	Cholesterol			% ester	Phospho-lipids	C/P	Tri-glycerides
			Total	Free	Ester				
PB	5	Control	446	127	319	72	397	1.12	111.5
	3	1 g/day	411	126	285	69	401	1.00	199.8
	4	1.5 "	351	121	229	65	421	.83	337.4
	8	Post Rx	418	120	298	71	368	1.14	106.5
ABB	4	Control	281	71	211	75	301	.93	108.9
	3	1.5 g/day	212	102	110	52	385	.55	513.0
	3	Post Rx	263	67	196	74	276	.95	96.6
GM	2	Control	257	67	189	75	288	.89	103.7
	3	1.5 g/day	204	71	133	66	293	.70	244.7
	4	Post Rx	243	65	178	73	291	.83	113.7

density lipoproteins) and >1.063 g/ml ("α" lipoproteins) (Table II) revealed that the esterified cholesterol content of both fractions was decreased.

Of great interest is the increase in serum phospholipid and triglyceride levels. Increase in serum phospholipids and decrease in serum cholesterol levels resulted in marked decrease in value for C/P ratio. Lipoprotein analysis revealed that the increase in serum phospholipids was due to increase in lipid P content of the lower density (<1.063 g/ml) lipoprotein fraction.

In subject PB, lipoproteins of density < 1.063 g/ml were further subdivided into fractions of density >1.019<1.063 g/ml (β lipoproteins) and density <1.019 ("low density β lipoproteins" plus very low density lipoproteins). Values are shown in Table III.

Analysis of β lipoprotein fraction revealed a diminished content of cholesterol and lipid P. In the lipoprotein fraction of density< 1.019 g/ml, sizeable increments in both cholesterol and lipid P content were noted. The portion of serum cholesterol and lipid P in this low density fraction had more than dou-

TABLE II. Cholesterol and Phospholipid Content (mg %) of Lipoprotein Fractions of Densities >1.063 (α) and <1.063 mg/ml.*†

Subject	Rx	Total cholesterol		% esterified		Phospholipid		C/P ratio	
		>1.063	<1.063	>1.063	<1.063	>1.063	<1.063	>1.063	<1.063
PB	Control	54	391	79	71	96	268	.50	1.34
	1 g/day	36	399	80	72	88	284	.41	1.41
	1.5 "	21	317	74	65	87	307	.24	1.03
	Post Rx	50	396	81	73	99	256	.51	1.55
ABB	Control	34	255	91	76	99	177	.35	1.45
	1.5 g/day	14	242	77	54	84	277	.17	.75
	Post Rx	48	234	81	75	106	158	.45	1.48
GM	Control	61	208	84	74	133	142	.46	1.45
	1.5 g/day	24	190	81	69	91	178	.26	1.07
	Post Rx	50	202	84	74	126	160	.39	1.27

* Cholesterol and phospholipid content of lipoproteins reported as mg lipid in designated lipoprotein fraction present in 100 ml serum.

† No. of samples of sera subjected to ultracentrifugal fractionation is the same as or slightly less than No. of samples listed for each subject in Table I.

TABLE III. Cholesterol and Phospholipid Content (mg %) of Lipoprotein Fractions of Densities >1.019 <1.063 and <1.019 (β) g/ml. Subject PB.

Rx	Total cholesterol		% esterified		Phospholipid	
	>1.019 <1.063	<1.019	>1.019 <1.063	<1.019	>1.019 <1.063	<1.019
Control	305	55	71	69	218	51
1 g/day	278	122	74	70	196	88
1.5 "	173	144	64	66	172	135
Post Rx	343	52	73	73	219	36

bled by the end of 14th day of treatment.

Summary. Administration of Benzmalecene alters serum lipids as follows: 1) decreases serum total and esterified cholesterol concentrations; 2) decreases cholesterol and phospholipid content of α (density >1.063 g/ml) and β (density $>1.019<1.063$ g/ml) lipoproteins; 3) increases serum phospholipid and triglyceride concentrations; 4) increases, both absolutely and relatively, cholesterol and phospholipid content of lipoproteins of density <1.019 g/ml; and, 5) decreases C/P ratio in whole serum and in α and β lipoproteins. The compound possesses extraordinary lipid-altering properties which warrant further investigation. However, the increase in low density lipoprotein lipids may be an undesirable effect since low density lipoproteins may play a role in atherogenesis.

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Received October 26, 1959. P.S.E.B.M., 1960, v103.

Effect of Phytic Acid on Zinc Availability.* (25498)

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Considering low zinc requirement of laboratory animals fed casein as source of protein it is surprising that, in recent years, deficiency symptoms have appeared in animals maintained under practical conditions(1). This has probably come about because of more common use of soybean meal in rations inasmuch as several investigators have found zinc in soy protein less available than that in animal proteins(2,3,4). Evidence has been presented that heat treatment increases availability of zinc in soy protein(5,6) although this observation has not been confirmed under our laboratory conditions. Phytin accounts for about 70% of phosphorus in soybean meal, and during extraction of soy protein, phytic acid forms a complex with protein(7). That isolated soy protein has a high affinity for zinc led us to test whether or not a casein-phytic acid complex would decrease availability of zinc to the growing chick.

Methods and material. To prepare casein-phytic acid complex, casein was suspended in distilled water to make a slurry, and phytic acid was added equivalent to 5% of dry weight. After standing overnight the mixture was dried in oven at 60°C. A similar complex was prepared with soy protein† using 4% of phytic acid. Phytic acid analyses were performed by the method of Pringle and Moran(8) except that samples were extracted twice for 18 hours. The original soy protein contained 0.5% of phytic acid phosphorus and the casein-phytic acid complex, 0.8%. According to analysis about one-fourth of phosphorus in commercial phytic acid was inorganic, and total phosphorus contents of proteins were: soy protein 0.9%; casein 0.7%; casein-phytic complex 1.8%. Basal diet, type of chicks and experimental conditions were as previously described(9) except that the battery was plastic coated. Other diets fed were modifications of the soy protein basal. Twenty-six % of soy protein-phytic acid complex, 18% of casein and 5% of gelatin, or 19% of casein-phytic acid complex and 5% of gelatin were substituted for soy protein in the respective diets and glucose hydrate was adjusted accordingly. Other additions were made at the expense of glucose.

Results of 2 trials are summarized in Table I. Rate of growth on casein-gelatin basal diet

* Contribution from Missouri Agri. Exp. Station, Journal Series No. 2066. Approved by Director.

†C-1 Assay Protein, Archer-Daniels-Midland Co., Cincinnati, O.

TABLE I. Availability of Zinc for Chick Growth.

Phytic acid-P in diet, %	Supplements	Wt at 4 wk (g) Trial 1	Trial 2
	A. Casein-gelatin basal*		
.0	None	460 (10)†	447 (10)
.0	Zn, 55 p.p.m.‡	469 (10)	446 (10)
.15	Casein-phytic acid complex	206 (10)	153 (7)
"	*Idem*, Zn, 55 p.p.m.	473 (10)	395 (9)
.29	1.2% Ca phytate§		438 (10)
"	*Idem*, Zn, 55 p.p.m.		476 (9)
	B. Soy-protein basal*		
.12	None	162 (9)	122 (9)
"	Zn, 15 p.p.m.	382 (9)	391 (9)
"	" 55 "	473 (10)	440 (9)
.26	Soy protein-phytic acid complex	97 (6)	94 (2)
"	*Idem*, Zn, 15 p.p.m.	227 (10)	201 (9)

* Zinc content of basal diets was 9 ± 0.5 p.p.m.
† Ten male crossbred chicks were started in each group. No. of survivors is shown in parentheses.
‡ Zinc added as $ZnCO_3$.
§ Nutritional Biochemicals Corp.

was not improved by zinc supplementation, and it may be concluded that approximately 9 p.p.m. of zinc as it occurs in casein-gelatin diet is adequate for growth of the chick. Growth on soy protein basal diet, which by analysis contained the same amount of zinc, was markedly improved by zinc supplementation. In agreement with earlier observations (9) addition of more than 15 p.p.m. of zinc was required to give maximum response on this type of diet.

When the casein-phytic acid complex and gelatin served as source of protein, growth rate was about the same as that on basal soy protein diet. These 2 diets contained approximately the same amounts of phytic acid phosphorus and zinc. Supplementation of casein-phytic acid diet with zinc supported near maximum rate of gain. In contrast to phytic acid in combination with casein, calcium phytate added to casein-gelatin basal diet had little or no effect on growth rate.

Chicks that received artificially prepared soy protein-phytic acid complex grew slowly and exhibited severe symptoms of zinc deficiency. When this diet, which contained 0.26% of phytic acid phosphorus as compared to 0.12% in basal soy diet, was supplemented with 15 p.p.m. of zinc, rate of growth was superior to that on basal diet. Thus 15 p.p.m. of zinc more than overcame the depressing effect of added phytic acid protein complex.

Although these results do not prove that the natural inhibitor in soy protein is phytic acid, they strongly suggest that phytic acid is involved in making zinc unavailable. Commercial soy protein used contains about 0.12% of phytic acid phosphorus, and a similar quantity of phytic acid complexed with casein makes zinc less available. The results suggest that phytic acid must be in combination with protein to make zinc unavailable, because addition of calcium phytate to the diet had little or no effect. It is known that phytic acid forms a complex with soy protein(7) and that proteins complexed with phytic acid are resistant to digestion by proteolytic enzymes such as pepsin(10). It is also possible that failure of calcium phytate to make zinc unavailable is due entirely to its insolubility.

Summary. Zinc in isolated soy protein is less available than that in casein. Zinc in a casein-phytic acid complex, which contains an amount of phytic acid comparable to that found in isolated soy protein, is also less available than that in untreated casein. Addition of calcium phytate to casein-gelatin type diet had little or no effect on zinc availability.

Authors gratefully acknowledge assistance of Dr. E. E. Pickett, Dept. of Agri. Chem., who performed zinc analyses by polarographic method, and A. E. Staley Manufacturing Co., Decatur, Ill., which supplied phytic acid.

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Received October 27, 1959. P.S.E.B.M., 1960, v103.

Factors Influencing Degree of Infectivity of Enterovirus Ribonucleic Acid.* (25499)

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I. *Reversibility of Infectivity of Poliovirus Ribonucleic Acid by Changes in Salt Concentration.* A number of investigators found that free ribonucleic acid (RNA) obtained from several different viruses exhibits only approximately 1/1,000th of the infectivity of intact virus from which it is derived. Fraenkel-Conrat(1) suggested that residual ribonuclease (RNAase) activity in his Tobacco Mosaic Virus RNA preparations accounts for at least some of the apparent instability of RNA, and that RNAase activity is particularly marked in isotonic solutions. Koch *et al.*(2) suggested that the lower infectivity of RNA as compared to whole virus might be due partly to a different and probably less efficient mechanism of entry of RNA into the cell. Our experiments were carried out to gain an understanding of factors which influence degree of infectivity of the active portion of RNA. Such information may be expected to throw some light on the explanation of the differential between infectivity titer of whole virus and that of RNA derived from it. This paper presents evidence that loss of infectivity of poliovirus RNA which occurs on dilution in isotonic salt solutions can be recovered quantitatively by addition of salt (NaCl and KCl).

Materials and methods. Infectivity test system. The cell line used throughout was the Fernandes passage line of human amnion cells (3) grown in Eagle's solution with twice the recommended concentration of amino acids and vitamins(4). RNA was inoculated on 3 day-old cells grown on 60 mm Petri dishes, and the plaque count was made 4 days later. Prior to inoculation with RNA the cells were washed 2 x with isotonic solutions. In all respects, other than use of isotonic wash solutions, the methods were identical to those described previously(5). *Solutions used.* "Minus" Hanks Tris (–HT) consisted of Hanks solution minus calcium, magnesium and phosphate, and buffered at pH 8 with Tris buffer. D_3T = 0.9 M NaCl, 8% sucrose, plus 0.1 M Tris buffer adjusted to pH 8 with HCl. E_1 virus is a preparation of partially purified (100 x concentrated) type 1 poliovirus received from Cutter Labs. It is said to have a molarity of approximately 0.88 with respect to NaCl. Infectious RNA was prepared by modification of phenol extraction method of Gierer and Schramm(6). This method has been described(5). *Alcohol Precipitation.* One part RNA was mixed with 2 parts 95% ethanol and kept at 4°C overnight. Usually no visible precipitation occurred, but since infectivity is regularly quantitatively recovered there seems reason to believe that RNA is precipitated by this process and adheres to the glass. The mixture was then centrifuged in a cold centrifuge at approximately 8,000 rpm for 30 minutes. The supernatant was discarded and glass-distilled water added to the residue in volume equal to that of original RNA preparation.

Results. An RNA preparation which has lost its infectivity in isotonic salt solution can regain its full degree of infectivity when diluted further in hypertonic salt solutions. Table I compares degree of infectivity of the same RNA preparation diluted in solutions of 3 different salt concentrations at constant pH. Two different RNA preparations were exam-

* Aided by grant from National Fn.

TABLE I. Effect of Molarity of Diluent on Degree of Infectivity of Ribonucleic Acid.

Origin of RNA	10^{-1} dilution made in	No. of plaques per plate, 10^{-1} dilution	Titer* on dilution in .9 M KCl + Tris	Titer* on dilution in .14 M –HT†
E_1‡ (.88 M)	.9 M KCl-Tris		1.9×10^4	0
	.14 M –HT		8×10^3	0
	D.W.§		1.3×10^4	0
E_1‡ (.88 M) Fresh RNA	.9 M KCl-Tris		1.6×10^4	
	D.W.	0, 0, 0	5×10^3	0
Frozen RNA	.9 M KCl-Tris		4×10^3	
	D.W.	0, 0, 2	4×10^3	0
Isotonic virus‖ (.14 M)	.9 M D_3T		approx. 5×10^5 (C)¶	
	.14 M –HT	0, 1, 0, 0	4.5×10^5 (C)	
	D.W.	0, 0, 0, 3	3.5×10^5 (C)	

* Plaque-producing particles/ml.

† –HT = Hanks Solution minus calcium, magnesium, phosphate + Tris buffer.

‡ E_1 fraction = Partially purified 100 × concentrated preparation of type 1 poliovirus (Mahoney strain), received from Cutter Labs; said to be .88 M with respect to NaCl.

§ D.W. = Glass-distilled water.

‖ Mahoney strain type 1 poliovirus grown in tissue culture in Scherer's Maintenance Solution + 10% horse serum; probably approximately .14 M.

¶ (C) = Confluent areas—counts partly estimated.

ined, one derived from the E_1 fraction of partially purified 100 x concentrated poliovirus, and one from crude tissue culture virus in isotonic media. Each was prepared by procedure described previously(5) and number of plaques/0.1 ml was measured after 10^{-1} dilution in 0.9 M KCl buffered with Tris,† 0.14 M "minus" Hanks solution (–HT) and glass-distilled water, all with pH approximately 8. These 10^{-1} dilutions of each RNA in the 3 different salt concentrations were further diluted to final concentration of 10^{-2} in 0.9 M KCl and Tris, and 0.14 M –HT and examined for plaque count. Table I shows that E_1 RNA, freshly prepared or frozen, or RNA from crude tissue culture virus in isotonic solution, if diluted 10^{-1} in distilled water or 0.14 M –HT saline only occasionally produces a plaque. However, when this non-infectious RNA is further diluted in hypertonic salt solution the original degree of infectivity can again be demonstrated.

In Table II are listed results of experiments on the influence of duration of exposure to hypotonic or isotonic environments on degree of recovery of infectivity when RNA so exposed is transferred to hypertonic salt solutions. The results listed in Table I were derived from experiments which had been carried out within a few minutes, and left open the possibility that irreversible damage to the RNA molecule might occur in hypotonic solutions if exposed for longer periods of time.

In spite of the long periods of exposure to isotonic or hypotonic environments, recovery of infectivity occurred regularly in hypertonic solutions. There is suggestive evidence that recovery was not always complete.

To reduce salt concentration to the lowest feasible degree and to eliminate the variable of small changes in salt concentration which existed in experiments in Table II (0.87 M experimental dilutions and 0.9 M controls), an RNA preparation made from a suspension of virus in isotonic medium was precipitated with alcohol, resuspended in distilled water, then exposed to distilled water in 10^{-1} dilution for periods varying from 1 to 24 hours. Low concentrations of salt were presumably still present but were probably less than 0.001 M. At each period shown in Table III the RNA was further diluted in D_3T so that final inoculum contained 0.9 M NaCl. Immediately and 60 minutes later, 0.1 ml was seeded on each of a series of monolayers. Where the difference in molarity between test and control RNA has been eliminated, no evidence was found of differences in degree of recovery of infectivity

† The 10^{-1} dilution in 0.9 M KCl buffered with Tris was not tested. Previous experience showed that this dilution regularly produced confluent cytopathogenic action.

TABLE II. Effect of Time Interval in Isotonic Solution on Titer of Ribonucleic Acid Further Diluted in Hypertonic Diluent.

Origin of RNA	Time diluted 10^{-1} in D.W.	No. of plaques in D.W.	Titer* on dilution in .9 M D_3T† Stat	30 min.	60 min.
E_1 (.88 M)	1 hr	0, 0	6.5×10^3		8.5×10^3
	3 "	0, 0, 0		4×10^3	7×10^3
	5 "	0, 0, 0		6×10^3	2.5×10^3
	Control		1.9×10^4		
Idem	1 hr	0, 0, 0	$>6.2 \times 10^4$(C)‡		$>9.5 \times 10^4$(C)
	5 "		4.5×10^4		6.8×10^4
	Control		6.2×10^4		
Isotonic virus (.14 M)	1 hr	0, 0, 1	$>1 \times 10^5$(C)	9.5×10^4	9.5×10^4
	Control		$>1.5 \times 10^5$(C)		
E_1 (.88 M)	1 hr	0, 0, 0	9×10^3	9×10^3	9×10^3
	3 "	0, 0, 0	4×10^3	2×10^3	5×10^3
	5 "	0, 0, 0	2×10^3	3.5×10^3	2.5×10^3
	Control		9×10^3		
Idem	1 hr	0, 0, 0, 0	2.5×10^2	1×10^3	1×10^3
	3 "	0, 0, 0, 0	1×10^3	1×10^3	1×10^3
	5 "	0, 0, 0	1.5×10^3	5×10^2	1.5×10^3
	Control		5×10^2		

* Plaque-producing particles/ml. † D_3T = .9 M with respect to NaCl. ‡ Confluent areas—count partly estimated.

of RNA as a result of different periods of time during which it was exposed first to distilled water, or subsequently to D_3T. Degree of infectivity recovered is not significantly different from degree found before dilution in distilled water.

Discussion. Loss of infectivity of RNA in distilled water, hypotonic or isotonic solutions and complete recovery of infectivity when the RNA is restored to a medium containing salt in hypertonic concentrations, has been shown to be a reproducible phenomenon. The significance of the phenomenon is clear only in that the reversibility of infectivity by hypertonic salt concentrations would seem to preclude participation of persistent RNAase in RNA preparations themselves as a major cause of inactivation in isotonic or hypotonic salt solutions. Inactivation of RNA infectivity by RNAase is not reversible.

TABLE III. Effect of Time Spent in Isotonic Solution on Titer of Ribonucleic Acid Further Diluted in Hypertonic Diluent at Intervals Noted.

	Time 10^{-1} dilution in D.W., hr	Titer on dilution in D_3T Stat	After 60 min.
Alcohol precipitated RNA from isotonic virus	1	1.1×10^5	1.4×10^5
	5	3×10^4	3×10^4
	18	5 "	9 "
	24	6 "	1.2×10^5
	Control	9 "	

Unexpressed infectivity of RNA may still be due to inactivation by RNAase of cells in test monolayers in low salt concentrations. Experiments to be reported later show that isotonic salt environments do enhance the effect of a host cell RNA inactivator, presumably RNAase. Preliminary observations suggest that RNA in low salt concentrations still does not produce infection despite reduction of the inactivator to undetectable levels by various means. The possibility that cell system inactivator plays an important part remains open, however. We have shown here only that RNAase plays no major role in inactivation of RNA in the diluent alone.

The theory proposed by Doty *et al.*(7) for the structure of RNA suggests that the molecule consists of a single strand of nucleoprotein with irregular hairpin-like helices along its length which straighten in iso- or hypotonic solutions and reform under hypertonic conditions. Infectivity responses to changes in molarity reported here may be the result of such changes in molecular structure. On the other hand, small changes in molarity which alter degree of infectivity can also be expected to change host cell surface permeability. Whether the salt acts on the host cell or on RNA itself, the data presented indicate that the RNA molecule in isotonic solutions is

still potentially biologically active; the expression of this activity is dependent on addition of salt.

Summary. In preparations of RNA made from polioviruses by phenol extraction technic, the infectivity of RNA which is rendered undetectable by dilution in isotonic or hypotonic salt solutions can be quantitatively recovered by addition of NaCl or KCl. This is true whether the exposure to low salt concentrations is brief, or as long as 24 hours. Reversibility of infectivity by hypertonic salt concentrations would seem to preclude participation of residual RNAase in the preparation itself as a major cause of inactivation of RNA infectivity with alterations in molarity.

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Received October 29, 1959. P.S.E.B.M., 1960, v103.

Effect of Vasopressin on Creatine Excretion in the Rat. (25500)

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In experiments designed to measure inhibition of postirradiation polydipsia and polyuria by antidiuretic hormone vasopressin, it was observed that 500 mU of Pitressin tannate in oil significantly increased urinary excretion of creatine in Co^{60} gamma irradiated rats as compared to irradiated non-injected controls(1). Evidence is presented here to show that a significant increase in urinary creatine excretion is also evoked in normal rats by injection of vasopressin.

Methods. Forty young female Wistar rats (135-282 g) were randomized by weight and injected intramuscularly with Pitressin tannate in peanut oil (Parke, Davis & Co.), aqueous Pitressin, commercial peanut oil (Planter's) or normal saline. Rats were housed in individual metabolism cages and fed Wayne Lab-Blox pellets except when noted; body weights, water intakes, and urine outputs were recorded daily. Twenty-four hour urine samples were analyzed for creatine by modification(1) of the method of Anderson *et al.*(2) and for creatinine by the Jaffe reaction after mechanically shaking (30 min) aliquots with 7 ml of Amberlite IRA-401 (30-60 mesh) in the chloride cycle (washed with 40 volumes of distilled water) and collecting the effluent by filtration over glass wool with 3 washes of distilled water. Recoveries of added creatinine averaged 90 $\pm$ 3%.

Results are summarized in Table I. In fed animals, significant creatinuria occurred on all 3 days after injection of 5 U Pitressin tannate in oil. Fasted control rats experienced the usual starvation creatinuria and Pitressin-evoked creatinuria was not significant until the second and third days. Of 5 rats injected with 20 U aqueous Pitressin, one died within 12 hours; in the remaining rats no significant increase in creatinuria was observed on either of 2 postinjection days. In experiment with fed rats, water intakes were significantly reduced on first postinjection day by Pitressin in oil ($P<0.01$) but no differences in water intakes were observed between fasted controls and fasted hormone-injected animals on first postinjection day. Water intakes of fed rats injected with aqueous Pitressin were not different from controls but urine outputs were significantly lower than controls on first postinjection day. In no experiment did body weights or creatinine excretions of hormone-injected rats differ from controls.

TABLE I. Average Daily Creatine Excretion (mg) and Urine Output (ml) of Normal Rats.

	0-24 hr		24-48 hr		48-72 hr	
	Cr*	Ur*	Cr	Ur	Cr	Ur
Fed rats inj. with 5 U Pitressin tannate in oil (10)	4.2 ± 2.1	5 ± 4	5.0 ± 2.4	4 ± 3	4.6 ± 2.3	4 ± 4
Fed rats inj. with peanut oil (10)	.9 ± .4	5 ± 4	1.4 ± .8	5 ± 4	2.1 ± 1.2	4 ± 4
P	<.01	NS	<.01	NS	<.02	NS
Fasted rats inj. with 5 U Pitressin tannate in oil (5)	6.6 ± 2.6	4 ± 1	8.2 ± 1.9	3 ± 1	7.8 ± 1.9	3 ± 1
Fasted rats inj. with peanut oil (5)	4.0 ± .7	3 ± 1	3.9 ± .8	3 ± 1	3.9 ± 1.1	3 ± 1
P	NS	NS	<.01	NS	<.01	NS
Fed rats inj. with 20 U aqueous Pitressin (4)	2.0 ± 1.1	8 ± 1	2.8 ± 1.9	11 ± 2		
Fed rats inj. with normal saline (5)	1.0 ± .1	12 ± 2	1.0 ± .2	9 ± 2		
P	NS	<.05	NS	NS		

* Cr, creatine; Ur, urine. No. in parentheses indicate No. of animals.

For this strain of rats with an average body weight of 165 ± 29 g, the average of 138 twenty-four hour urinary creatinine determinations was 2.7 ± 1.2 mg.

Discussion. Aqueous solutions of vasopressin when injected intramuscularly are absorbed into the blood stream very rapidly and it has been shown that aqueous Pitressin in concentrations of 100 mU/100 g, when injected intravenously in anesthetized rats is cleared (inactivated) from blood within 5 min, primarily by kidney and splanchnic vascular bed(3). Since aqueous solutions of the hormone did not evoke creatinuria, it is concluded that a sustained elevation of blood level of vasopressin is necessary for the effect. Duration of creatinuria after injected Pitressin tannate in oil is at least 4 days, as judged by subsequent experiment in which significant creatinuria was observed for this length of time. This duration is in approximate agreement with the observation that antidiuresis in the hydrated rat is detectable for several days after injection of comparable doses of Pitressin tannate in peanut oil(4). If the reasonable assumption is made that the 5 U in our experiments were released from injection site at an *average* rate of 50 mU/hour into blood volume of 10 ml, then the observed creatinuria probably results from an *average* blood level of about 5 mU/ml. This concentration greatly exceeds the physiological level of the antidiuretic hormone, estimated to be 0.8 to 3.0 μU/ml(5), but is comparable in order of magnitude to concentrations of 10-20 mU/ml found after massive hemorrhage(6).

These results suggest that creatinuria resulting from forms of stress such as lethal irradiation and starvation may be mediated by a sustained elevation of the antidiuretic hormone. Although the least dose of Pitressin capable of producing the effect in normal rats has not been established, it is probable (assuming adding of effects on creatine excretion of irradiation and Pitressin(1)) that the threshold dose is lower than that reported here by a factor of at least 10.

Summary. Intramuscular injection of 5 U of the antidiuretic hormone vasopressin (Pitressin tannate) in oil evoked a significant creatinuria for 72 hours in fed rats and a significant creatinuria from 24-72 hours in fasted rats, without affecting excretion of creatinine. Aqueous solutions of the hormone (20 U) did not produce significant creatinuria.

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Received October 29, 1959. P.S.E.B.M., 1960, v103.

Staphylococcal Immunity: Production of Staphylococcal Hemagglutinins in Rabbits Receiving Staphylococcal Vaccine.* (25501)

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Patients with focalized staphylococcal disease often fail to demonstrate significant humoral antibody against staphylococci or staphylococcal products. Neter and his associates have recently demonstrated that hemagglutination technics are of sufficient sensitivity to allow detection of antistaphylococcal antibody in human gamma globulin and to a lesser extent in pooled human sera (1). The present study was undertaken to determine the nature and extent of serologic response of rabbits to a staphylococcal bacterial cell vaccine, utilizing a hemagglutination test for measurement of serum antibody. The vaccines employed, the methods evolved for performing hemagglutination tests, antigens used in the procedure, and the nature of antibody response are reported here.

Materials and methods, vaccines. The Giorgio strain of staphylococcus was used for preparation of the 2 vaccines employed.‡ *One vaccine* was prepared from surface growth obtained on 15 cm meat infusion agar plates heavily seeded with 0.85% saline suspension prepared from 18 hour culture of staphylococci. Following incubation for 18 hours at 37°C, 10 ml saline was added to each plate, and the thick suspension obtained was pooled and centrifuged at 1200 rpm for 1 hour. The sedimented microorganisms were twice washed with 70 ml saline and resuspended in 200 ml saline. The resultant homogeneous suspension containing approximately 10 billion microorganisms/ml was placed in 60 ml rubber stopper bottles and sterilized by heating in 60°C. water bath for one hour on 3 separate days. The pH of final vaccine was 6.9. *A second vaccine* was prepared from 4 hour cultures. Culture plates similarly seeded were harvested after 4 hours incubation at 40°C. The growth obtained was removed in 125 ml of hemagglutinin buffer (Difco pH 7.3) and well shaken in Erlenmeyer flask containing glass beads. The bacterial mass obtained from such young cultures was viscous and slimy, which made washing difficult. After centrifugation, most of supernatant was removed, and an attempt made to produce a homogeneous suspension from the glutinous material by 2 additional washings with 120 ml buffer. The resultant volume of bacterial cells was suspended in 175 ml of buffer in 500 ml Erlenmeyer flask, and the preparation heated at 60°C for one hour with constant agitation. During this heating, the viscosity gradually disappeared. This preparation was pipetted into 60 ml bottles, shaken, and reheated at 60°C for 15 minutes to attain sterility. The pH of final vaccine was 7.3. *Immunization of rabbits.* Male albino rabbits weighing 4 to 5 kilos were used. Animals were housed in individual cages and received rabbit pellets, lettuce, carrots, and water *ad lib.* In initial experiments, 4 rabbits were injected at intervals of 7 to 10 days with increasing amounts of 18 hour vaccine (0.2 to 1.0 ml). A different route of administration (intravenous, subcutaneous, intraperitoneal, and intradermal) was used in each rabbit using equal total quantities of vaccine. A second series of 6 rabbits were also injected by various routes with increasing doses of 4 hour vaccine. The first injection was 0.5 ml, each succeeding injection increasing by 0.5 ml to sixth and final injection of 3 ml. Two rabbits received vaccine intravenously, 2 subcutaneously, and 2 intraperitoneally. Rabbits were bled from the ear prior to first injection and at appropriate intervals thereafter. Sera were

* This study supported in part by research grants from Nat. Inst. of Allergy and Infect. Disease and Nat. Heart Inst., Bethesda, Md., and by grants from Chas. Pfizer & Co., Brooklyn, N. Y., Upjohn Co., Kalamazoo, Mich., and Wyeth Labs., Philadelphia, Pa.

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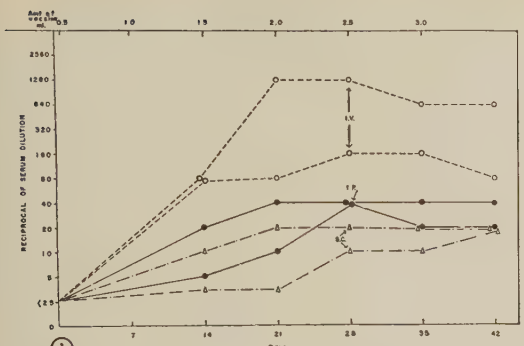
‡ This phage group III is lysed by bacteriophages 47/53/54/75/VA4, and has been used extensively in experimental studies within this institution(2).

stored at –20°C and inactivated at 56°C for 30 minutes before use. *Staphylococcal antigens.* Four different antigenic preparations were prepared from the Giorgio strain grown as already described. Antigen A was prepared from 18 hour vaccine used for immunization of the first group of rabbits. The vaccine was centrifuged at 2,000 rpm for 2 hours and the slightly cloudy supernatant was used directly as the antigen. Antigen B differed only in that the washed staphylococci were killed by boiling for 1 hour prior to centrifugation. Antigen C was prepared from 4 hour cultures. The bacteria were recovered from plate surfaces in buffer, shaken in an Erlenmeyer flask without washing, and heated to 60°C for 15 minutes. Following centrifugation at 2,200 rpm for 2 hours, the sterile yellow supernatant obtained was utilized as antigen. Antigen D: Three separate crude polysaccharide preparations were made from the Giorgio strain utilizing the method of Keogh, North, and Walburton(3). The first and most potent preparation was prepared from 18 hour cultures. After centrifuging to remove a fine precipitate, the final product was dissolved in Hanks' solution. This crude polysaccharide was slightly opalescent. The other 2 preparations were prepared from twice washed 4 hour cultures, and the gelatinous sediments were used for preparation of polysaccharide antigen. These final products dissolved in buffer solution were water clear after centrifugation. Bacterial antigens were also prepared from 5 heterologous strains of heat killed coagulase positive staphylococci in the same manner as outlined for antigen B. These microorganisms had all been isolated from human infections, produced yellow pigment, hemolysins, and fermented mannite. They belonged to each of the 4 phage groups and were lysed by the following bacteriophages: Strain 1, 29; Strain 2, 55/3A/3B/3C; Strain 3, 6; Strain 4, 42B; Strain 5, 80/81. In addition, one coagulase negative strain (MAM), which has been utilized extensively in our other studies(4), was similarly utilized in production of antigen. *Hemagglutinin tests.* Polyphil sheep red cells§ were washed 3 times with saline or Difco hemagglutinin buffer. (In the first experiments, saline was used to wash and suspend the red cells, subsequently only buffer was used.) To washed packed red cells in Erlenmeyer flask, undiluted antigen was added sufficient to make a 2.5% or 1.25% red cell suspension. The suspension was thoroughly shaken and incubated at 37°C for 40 minutes. The modified red cells thus obtained were centrifuged, washed 3 times with 10 ml buffer, and resuspended in 9.75 ml of buffer. In experiments in which 1.25% red cells were utilized, an aliquot of the modified suspension of 2.5% red cells was diluted 1 to 2 with buffer to obtain a 1.25% red cell concentration which had been exposed to the same amount of antigen. Serums from normal or immunized rabbits were serially diluted with buffer (or saline) and 0.2 ml of each dilution pipetted into 7.5 × 0.8 cm agglutination tubes. 0.2 ml of washed 2.5% or 1.25% modified red cells was added to each tube and tubes immediately mixed. All tubes were incubated at 37°C for 40 min. The resulting agglutination reactions were read grossly after centrifugation at 1000 rpm for one minute. *Controls* of modified red cells and buffer, normal red cells and buffer, and inactivated immune sera and normal red cells were included in each test. Control tubes containing normal red cells and buffer were consistently negative. Six of 10 sera obtained from 10 normal rabbits showed hemagglutination at a dilution of 1:2, all sera were consistently negative at greater dilution. Conversely, 2 of 6 immune sera titrated with normal red cells showed hemagglutination in the initial 1:2 tube, but all were negative at higher serum dilution.

Results. 1. *Characteristics of hemagglutinin response to vaccination.* Rabbits receiving staphylococcal bacterial cell vaccines prepared from 4 to 18 hour cultures responded to immunization in a predictable manner. Hemagglutinins were generally detectable at initial bleeding 14 to 22 days following start of weekly vaccinations, rising to maximal titers at 30 to 40 days. Hemagglutinin titers then remained relatively constant or declined slowly over 42 to 98 days observation, despite

§ Obtained weekly from Carworth Farms, New City, N. Y.



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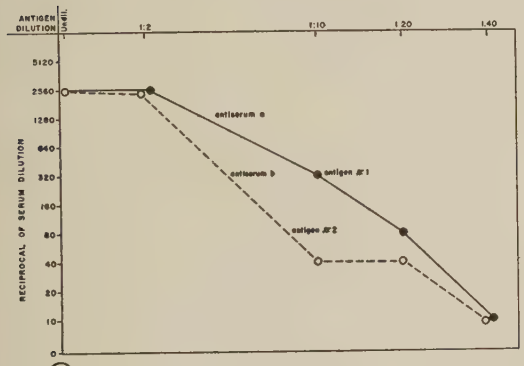

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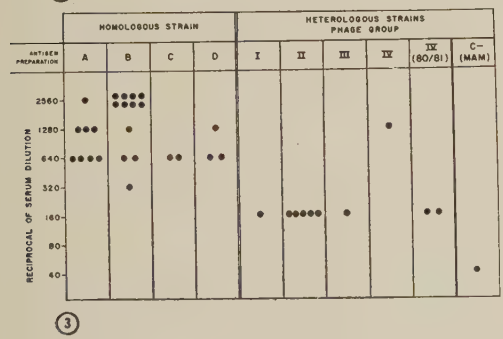

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FIG. 1. Hemagglutinating antibody response to staphylococcal vaccine.

FIG. 2. The influence of antigen dilution on the detection of hemagglutinating antibody.

FIG. 3. Hemagglutination titers obtained with different antigen preparations tested against a single high titer serum.

weekly injections of increasing quantities of vaccine.

In general, the intravenous route appeared to provoke a swifter hemagglutinin response which rose to higher levels than was seen following vaccination by intracutaneous, subcutaneous, or intraperitoneal routes. One experiment in which 6 rabbits were injected weekly *via* different routes with increasing amounts of 4 hour bacterial cell vaccine is pictured in Fig. 1. Hemagglutinin titers recorded represent serum dilutions at which 2+ or greater hemagglutination reactions were observed.

2. *Effect of variations in concentration of sheep erythrocytes on hemagglutinin titer.* In experiments performed against a serum of high titer it was found that 1.25% red cell concentration yielded optimal results. Endpoint titers were consistently 1 or 2 tubes higher utilizing this concentration of cells than those obtained with a standard 2.5% red blood cell suspension.

3. *Influence of antigen concentrations of hemagglutinin titer.* Variations in concentration of antigen used in sensitizing red blood cells produced marked differences in subsequent demonstration of hemagglutinin. Dilutions of antigen greater than 1:2 consistently reduced sensitivity of the hemagglutinating test procedure. A typical experiment is pictured in Fig. 2. Progressive dilution of 2 different antigens resulted in progressive decline in apparent antibody titer from 1:2560 to 1:10 when sensitized red cells were subsequently tested against 2 sera of high titer. Undiluted antigen was thus employed in standard test procedure.

4. *Influence of variations in antigen on detection of hemagglutinin.* Hemagglutination reactions obtained with 4 different Giorgio antigen preparations indicated that, in general, antigens prepared from supernatants from heat-killed 18 hour cultures produced hemagglutinin titers of the same order (A and B, Fig. 3). Antigens prepared from heat-killed 4 hour cultures (C, Fig. 3) and crude polysaccharide preparations (D, Fig. 3) produced a 2 to 4 fold reduction in apparent end point titers, but these differences were not of statistical significance and suggested that these preparations contained similar antigenic products.

Use of supernatants from living 4 hour cultures consistently produced hemolysis of red blood cells, thus rendering such antigens unsatisfactory for test procedure. In contrast, no hemolysis was obtained when 18 hour culture supernatants were employed.

Study of this phenomenon suggested that

such hemolysis resulted from presence of staphylococcal exotoxin in young culture preparations. Four hour cultures were consistently of lower pH (7.4) than 18 hour cultures (pH 8.2 to 8.4), suggesting that exotoxin was inactivated by progressive alkalinization of older cultures. Growth of staphylococci in 20% CO_2 for 18 hours with maintenance of pH 7.4, known to increase the yield of staphylococcal exotoxin(5,6), resulted in striking hemolysis of red blood cells when such 18 hour cultures were used for sensitization. Thus production of a satisfactory antigen required either prolonged growth or heating to inactivate the hemolysin produced by growing staphylococci.

Antigens prepared from 5 heterologous strains of coagulase-positive staphylococci yielded, with one exception, definite but significantly lower titers of hemagglutinating activity against a standard high titer serum. The single coagulase negative strain showed a low but definite cross reactivity. These results are portrayed in Fig. 3.

Discussion. Studies on staphylococcal immune mechanisms in the past have been hampered by lack of a test procedure of sufficient sensitivity to allow detection of antibody response to staphylococcal infection or staphylococcal vaccine preparations. Our experiments indicate that variations in concentration of red blood cells or antigens employed in the procedure produced striking alterations in results obtained, rendering careful standardization of the test procedure necessary for reproducible results. Similarly, staphylococcal exotoxin elaborated by certain strains required inactivation by heat or prolonged growth to prevent interfering hemolysis in the test system. The hemagglutination test appears to offer promise as a sensitive system for detection of antibody substances produced in response to certain staphylococcal products.

Summary. 1. Injections of 2 staphylococcal bacterial cell vaccines resulted in prompt antibody response in normal adult rabbits which could be detected by a simple hemagglutination test. Maximal antibody titers were reached in 30 to 40 days with weekly injections. The intravenous route yielded maximal antibody production. 2. Variations in concentration of red blood cells or antigen employed in sensitizing red cells produced marked alterations in the hemagglutinating end point when tested against standard high titer sera. Careful standardization was thus required to obtain reproducible results. 3. Sensitizing antigen prepared from various heterologous coagulase positive staphylococci of different phage types yielded cross reactions at lower dilutions against sera obtained from vaccinated animals. Antigen prepared from a single coagulase negative strain cross reacted with high titer serum only at low dilutions.

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Lipemia-Producing Activity of Pituitary Gland: Separation of Lipemia-Producing Component from Other Pituitary Hormones.* (25502)

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Our previous reports(1,2,3) described lipemia-producing activity of a crude extract derived from human, sheep, beef or hog pituitary glands. The lipemia-producing activity was present largely in anterior lobe of the gland(2). The 6 recognized anterior lobe hormones, when tested individually, had no effect upon rabbit's serum lipids(2). It was therefore concluded that lipemia-producing activity of crude pituitary extract is caused by the synergistic action of 2 or more of the recognized pituitary hormones, and/or by a pituitary substance which is different from recognized pituitary hormones. Our purpose was to identify the substance or substances in the pituitary gland, responsible for the lipemic effect. This report presents evidence that the pituitary gland contains a lipemia-producing component which is different from the recognized pituitary hormones. Concentration of this component into a fraction ("Fraction H") which is free from recognized pituitary hormones is described. Lipemia-producing activity of Fraction H is potentiated by simultaneous injection of ACTH‡ Production of lipemia in the rabbit by simultaneous injection of ACTH and commercial preparations of TSH, prolactin, or FSH, is also described. A possible relationship between synergism of ACTH with Fraction H, and synergism of ACTH with commercial preparations of TSH, prolactin and FSH, is discussed.

Materials and methods. Lyophilized intact hog pituitary glands were obtained from Armour Labs. Commercial preparations of anterior pituitary hormones, obtained from Armour and Wilson Labs, were derived from pituitary glands of the following species: ACTH, from hog pituitaries; prolactin and FSH, from sheep pituitaries; GH and TSH, from beef pituitaries; ICSH, from horse pituitaries. IRC-50 (CG-50, Type 2) cation exchange resin, furnished by Rohm & Haas Co., was prepared for use by the method of Hirs *et al.*(4). Lipemia-producing activity of various pituitary fractions was assayed, as previously described(2), by measuring change in serum total lipids of the rabbit 18 hours after a single subcutaneous injection. Other pituitary hormonal activities were assayed by methods described as follows: ACTH(5), GH (6), TSH(7), prolactin(8), ICSH(9), FSH (10), oxytocin and vasopressin(11).

Results. Variation in serum lipid level of rabbits treated with a single subcutaneous injection of H_2O was first studied to establish what magnitude of change in serum lipid level of rabbits treated experimentally with pituitary material should be considered statistically significant. The results are shown in Table I. Average change in serum lipid level of 15 rabbits treated with H_2O was +50 mg%. Estimated standard deviation of the change was $\pm$ 142 mg %. In subsequent experiments, the statistical significance of changes in serum lipid level of rabbits treated with pituitary material was evaluated by comparison with findings in the group of rabbits treated with H_2O. Thus, in a group of 4 rabbits injected with experimental material, an increase in the group's average serum lipid level which was less than 380 mg% was considered statistically not significant ($p>0.01$).

The effects upon rabbit's serum lipid level of commercial preparations of the 6 recognized anterior lobe hormones, when tested separately, are described in Table I. Dosages at which ACTH, GH, and TSH were tested

* This investigation was supported by research grants from N. Y. Heart Assn. and Nat. Heart Inst., N.I.H.

† Senior Research Fellow, N. Y. Heart Assn.

‡ The following abbreviations are used: ACTH, adrenocorticotropin; GH, growth hormone; TSH, thyroid-stimulating hormone; ICSH, interstitial-cell stimulating (luteinizing) hormone; FSH, follicle-stimulating hormone.

TABLE I. Effect of Various Pituitary Fractions upon Serum Lipid Level of Rabbit, Which Was Measured before, and 18 Hours after, Single Subcutaneous Injection. Following hormone preparations were employed: ACTH, Wilson #104746; GH, Armour #R50109; TSH, Armour #T3308; Prolactin, Armour #U10303; ICSH, Armour #T10812; FSH, Armour #R19911.

Material inj.	No. rabbits	Change in serum total lipid (mg %)
5 ml distilled H_2O	15	+ 50 ± 35*
60 mg lyophilized hog pituitary glands	5	+1450 ± 205
120 units ACTH	5	+ 10 ± 69
12.5 mg GH	5	+ 10 ± 54
25 " "	8	+ 190 ± 64
10 units TSH	7	+ 60 ± 30
25 mg Prolactin	5	+ 110 ± 48
25 " ICSH	6	− 40 ± 64
25 " FSH	8	+ 20 ± 39
60 units ACTH		
+ 6 mg GH	5	+ 270 ± 144
+ 4.6 units TSH	5	+1050 ± 220
+ 12.5 mg Prolactin	4	+ 810 ± 376
+ " " ICSH	4	+ 320 ± 251
+ " " FSH	4	+ 520 ± 233
6 mg GH		
+ 4.6 units TSH	5	+ 130 ± 37
+ 12.5 mg Prolactin	4	+ 100 ± 61
+ " " ICSH	4	+ 70 ± 96
+ " " FSH	4	+ 30 ± 27
4.6 units TSH		
+ 12.5 mg Prolactin	4	+ 100 ± 134
+ " " ICSH	4	+ 130 ± 226
+ " " FSH	4	+ 130 ± 146
12.5 mg Prolactin		
+ 12.5 mg ICSH	4	+ 210 ± 90
+ " " FSH	4	+ 30 ± 52
12.5 mg ICSH + 12.5 mg FSH	4	+ 10 ± 45

* Avg and stand. error of change in serum total lipid of group.

separately represent amount of each hormone present in 0.5 g or more of desiccated pituitary gland. Prolactin, ICSH and FSH were tested at a dosage of 25 mg§. At these dosage levels, these hormones did not have a statistically significant effect upon rabbit's serum lipid level. Table I also shows marked increase in rabbit's serum lipid level produced by a single injection of an alkaline extract of 60 mg of desiccated hog pituitary gland.

The effect upon rabbit's serum lipid level of injection of various combinations of commercial preparations of the 6 anterior lobe hormones is also shown in Table I. Dosages at which ACTH, GH and TSH were tested in combination, represent amount of each hormone present in 0.2 g of desiccated hog pituitary gland. Prolactin, ICSH and FSH were tested in combination at a level of 12.5 mg§. Table I shows that when ACTH was injected together with TSH, prolactin or FSH, a statistically significant increase was produced in average serum lipid level of treated rabbits. The combinations ACTH + GH, ACTH + ICSH, and the various combinations which did not include ACTH, did not have a statistically significant effect upon rabbit's serum lipid level.

The effect of the combination ACTH + TSH was then reexamined, in experiments which employed 2 different purified preparations of TSH supplied by Dr. R. W. Bates (Nat. Inst. Health). Dosages of ACTH and purified TSH were the same as in experiments described in Table I. The combination ACTH + purified TSH did not have a statistically significant effect upon average serum lipid level of treated rabbits. This lack of effect of the combination ACTH + purified TSH indicates that the lipemic effect of the combination ACTH + commercial TSH shown in Table I, is largely due to synergism of ACTH with some "contaminating" substance in the commercial TSH preparation, rather than to synergism of ACTH with TSH itself. The possibility of a similar basis for the effects of ACTH + commercial prolactin, and ACTH + commercial FSH, has not yet been investigated.

Information was now sought as to whether the synergistic action of ACTH with other pituitary substances is responsible for the entire lipemia-producing activity of crude pituitary extract. In preliminary experiments(12), a crude saline extract of hog pituitary glands was fractionated by the method of Bonsnes and White(13). Lipemia-producing activity was found only in material precipitated between 50% and 90% acetone concentration ("acetone 50-90% fraction"). About one-half of the lipemia-producing activity of pituitary glands used as starting material was re-

§ Concentration of these hormones in desiccated hog pituitary glands is uncertain.

covered in the acetone 50-90% fraction. The major portion of recovered lipemia-producing activity was found in the acetone 75-90% fraction(12). Bonsnes and White demonstrated that the acetone 50-90% fraction is free of ACTH, GH, and prolactin(13). They also showed that the acetone 75-90% fraction is free of TSH as well(13). These hormones are precipitated in earlier fractions in the Bonsnes-White procedure. These observations indicate that the synergistic action of ACTH is responsible for only a part of the total lipemia-producing activity of the pituitary gland, and demonstrate the presence, in acetone 75%-90% fraction, of a lipemia-producing substance which is different from ACTH, GH, TSH and prolactin. A fractionation method, based on Bonsnes-White procedure, was then devised for concentration of this lipemia-producing substance. This method is described below.

All operations are carried out at 0-5°C. Five g of lyophilized intact hog pituitary glands are pulverized and extracted for 4 hours with 150 ml 2% NaCl solution, the extraction mixture being maintained at pH 8.5 by periodic addition of 1 N NaOH. The insoluble material is removed by centrifugation and discarded. The supernatant is adjusted to pH 4.3 by dropwise addition of 0.4 N HCl. The precipitate is removed by centrifugation and discarded. The supernatant solution is adjusted with H_2O to a volume of 100 ml and an equal volume of acetone is added with constant stirring for 5 minutes. The precipitate (acetone 0-50% fraction) is removed by centrifugation and discarded. To the supernatant solution, 200 ml of acetone is added with constant stirring for 5 minutes. The precipitate (acetone 50-75% fraction) is again removed by centrifugation and discarded. To the supernatant solution, 600 ml of acetone is added with constant stirring for 5 minutes. The precipitate (acetone 75-90% fraction) is collected by centrifugation and dissolved in 40 ml H_2O. This solution is adjusted to pH 5.80, dialyzed 8 hours against H_2O and lyophilized. The yield of lyophilized material, called Fraction G, is 15 mg from each gram of desiccated pituitary glands.

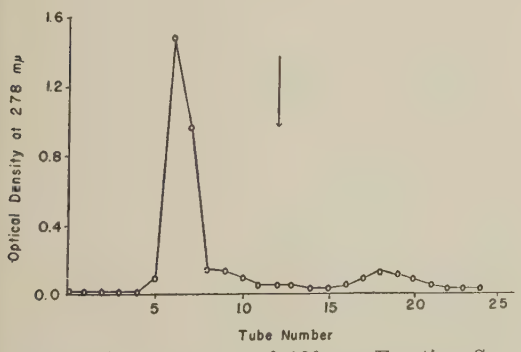

FIG. 1. Chromatogram of 100 mg Fraction G on 2.2 × 30 cm column of IRC-50 resin. Effluent collected in 10 ml fractions. Initial solvent, 0.20 M sodium phosphate, pH 5.80. At arrow, solvent changed to 1.0 M NaCl. Temp. 25°.

TABLE II. Effect of Fraction H upon Serum Lipid Level of Rabbit. Serum total lipid was measured before, and 18 hours after, a single subcutaneous injection.

Material inj.	No. rabbits	Change in serum total lipid (mg %)
Fraction H 1.5 mg	4	+ 240 ± 50*
2 "	7	+ 510 ± 164
3 "	4	+1010 ± 142
5 "	5	+2080 ± 176
0.5 mg Fraction H + 60 units ACTH†	8	+ 500 ± 162
1 mg Fraction H + 60 units ACTH	5	+ 790 ± 241
120 units ACTH	5	+ 10 ± 69

* Avg and stand. error of change in serum total lipid of group.

† ACTH preparation employed was Wilson #104746.

One hundred and twenty mg of Fraction G is dissolved in 15 ml of 0.2 M sodium phosphate buffer, pH 5.80. Insoluble material is removed by centrifugation and discarded. The supernatant solution is placed on 2.2 x 30 cm column of IRC-50 resin, which has been equilibrated to pH 5.80 with 0.2 M sodium phosphate buffer.‖ Operation of the column is described in Fig. 1. The effluent is collected in 10 ml fractions, and protein content of each fraction is estimated from optical density at 278 mμ. Lipemia-producing activity is concentrated in the first fraction (tubes 5 through 10), which contains about 30% of the material placed on the column. These tubes are pooled and $(NH_4)_2SO_4$ added to give a

‖ Before use the column is washed with buffer until pH of effluent is 5.80.

concentration of 42 g % of $(NH_4)_2SO_4$. The resulting precipitate is collected by centrifugation, dissolved in H_2O, dialyzed for 8 hours against H_2O and lyophilized. The yield of lyophilized material, called Fraction H, is 5 mg/g of desiccated pituitary glands.

Fraction H was assayed for lipemia-producing activity and also assayed for other pituitary hormonal activities. The effect of Fraction H upon rabbit serum lipid level is described in Table II. When injected separately at a dose of 3 mg, Fraction H caused an average increase of 200% in rabbit serum lipid level.¶ When injected with 60 units of ACTH, the effect of Fraction H was intensified. As little as 0.5 mg of Fraction H, when injected with 60 units of ACTH, caused a statistically significant increase in average serum lipid level of treated rabbits.

Fraction H was assayed for other pituitary hormonal activities.** The results in Table III show that none of the recognized anterior or posterior pituitary hormones is present in Fraction H in a proportion of more than 0.8% by weight. To determine whether the lipemia-producing activity of Fraction H might be due to the combined effects of these minute amounts of recognized pituitary hormones, a mixture of recognized hormones was made according to maximal proportions in which they can be present in Fraction H. This mixture was injected into rabbits at various dosages, the highest dose containing maximal amount of each pituitary hormone which can be present in 25 mg of Fraction H. No statistically significant effect upon rabbit serum lipid level was observed.

TABLE III. Results of Bioassays of Fraction H. Left-hand column shows hormonal assays performed. Middle column gives result of each assay. Right-hand column gives proportion in which each of pituitary hormones is present in Fraction H. These proportions were calculated by comparing hormonal activities of Fraction H with activities of highly purified preparations of each of the pituitary hormones.

Hormonal activity assayed	Hormonal activity of Fraction H	Proportion in which each pituitary hormone is present in fraction H (%)
ACTH	<.04 USP units/mg	<.03
GH	<.01 *idem*	<.8
TSH	.01 "	.07
Prolactin	<.0002 I.U./mg	<.001
ICSH	No activity detected at total dose of 4 mg	<.5
FSH	No activity detected at total dose of 2.5 mg	<.06
Oxytocin	.012 unit/mg	.002
Vasopressin	<.010 "	<.004

Discussion. By modification of the Bonsnes-White fractionation method, a pituitary fraction (Fraction H) has been prepared which is highly active in producing lipemia in the rabbit. Three mg of Fraction H has approximately the same effect upon rabbit serum lipid level as an alkaline extract of 60 mg of lyophilized hog pituitary glands (Tables I, II). Results of biological assays show that none of the recognized anterior or posterior pituitary hormones is present in Fraction H in a proportion of more than 0.8% by weight. A mixture containing maximal amounts of the recognized pituitary hormones which can be present in 25 mg of Fraction H had no effect upon rabbit serum lipid level. These observations indicate that Fraction H contains a lipemia-producing substance which is different from the recognized pituitary hormones.

Only a small proportion (about 10%) of lipemia-producing activity of hog pituitary glands is recovered in Fraction H. This low recovery may result either from losses of the lipemia-producing substance in fractions dis-

¶ Chemical analyses of sera obtained from rabbits before and after injection of Fraction H show that the increment of serum lipids following injection is composed largely of triglycerides (85%), together with small proportions of cholesterol (5%) and phospholipids (10%). Serum lipid level begins to increase 6 to 10 hours after the injection and remains elevated for 12 to 24 hours. Serum nonesterified fatty acid level increases markedly within 1 hour after the injection and remains elevated for 12 to 24 hours.

** Assays were performed by the following: ACTH assay by C. N. Mangieri (South Mountain Labs., Maplewood, N. J.); GH assay by A. E. Wilhelmi (Emory Univ.); TSH assay by R. W. Bates (Nat. Inst. Health); prolactin assay by I. Levenstein (Leberco Labs., Roselle Park, N. J.); ICSH assay by S. J. Segal (Rockefeller Inst.); FSH assay by S. L. Steelman (Merck Inst.); oxytocin and vasopressin assays by F. C. Armstrong (Parke, Davis & Co.).

carded during fractionation procedure, or from partial inactivation of the lipemia-producing substance during fractionation. It must also be caused in part by removal of other pituitary hormones, especially ACTH, which may act synergistically with each other or with Fraction H to raise serum lipid level of the rabbit.

Interpretation of the lipemic effect produced by simultaneous injection of ACTH with certain preparations of TSH, prolactin or FSH (Table I), is complicated by the impure nature of commercially available hormone preparations employed. Two possible hypotheses to explain observations in Table I are suggested: ACTH may act in synergism with TSH, prolactin or FSH to increase circulating lipids of the rabbit; or ACTH may act in synergism with some "contaminating" substance present in commercially available preparations of these 3 hormones.

The synergistic action of ACTH upon rabbit serum lipids was greatest when ACTH was combined with the commercial preparation of TSH (Table I). Combination of ACTH with more highly purified TSH preparations did not have a statistically significant effect upon average serum lipid level of treated rabbits. This observation shows that, in the case of the combination ACTH + commercial TSH, ACTH acts in synergism principally with a "contaminating" substance in the TSH preparation, rather than with TSH itself. Similar studies, employing more highly purified preparations of prolactin and FSH, will be required to determine whether the apparent synergism between ACTH and each of these hormones, may also be due to presence of a "contaminating" substance in commercial preparations of these 2 hormones.

The observation that the lipemia-producing activity of Fraction H is enhanced by simultaneous injection of ACTH may provide a clue to the identity of the hypothetical "contaminating" substance in commercial preparations of TSH, prolactin and FSH. It is possible that the synergistic action of ACTH with commercial preparations of TSH, prolactin and FSH may be caused in whole or in part by presence in these preparations of small amounts of the same lipemia-producing substance, concentrated in Fraction H.

Our experiments provide no information concerning the mechanism by which ACTH exerts its synergistic action upon lipid metabolism of the rabbit. This action may be related to other "extra-adrenal" metabolic effects of ACTH(14), or it may depend upon stimulation of adrenal cortical secretion by ACTH.

These observations show that lipemia-producing activity of crude hog pituitary extract is caused by the combined actions of (a) lipemia-producing component in Fraction H; (b) ACTH, and (c) substances present in commercial preparations of TSH, prolactin and FSH. Further studies will be required before it will be possible to compare the contributions of each of these pituitary components to total lipemia-producing activity of the pituitary gland.

Summary. A method for fractionation of hog pituitary glands by saline extraction, fractional precipitation with acetone at pH 4.3, and ion-exchange chromatography, is described. The final fraction has high lipemia-producing activity in the rabbit and is free of recognized pituitary hormones. Lipemia-producing activity of this fraction is enhanced by simultaneous injection of ACTH. Production of lipemia in the rabbit by injection of ACTH together with commercial preparations of TSH, prolactin or FSH is also described. In the combination ACTH + commercial TSH, evidence is presented that ACTH acts in synergism with some "contaminating" substance in commercial TSH preparation, rather than with TSH itself.

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Interpretation of the Master "2-Step" Exercise Electrocardiogram by Quantitative Analysis of RS-T Segment. (25503)

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This study was undertaken to formulate additional criteria for interpretation of the Master "2-Step" exercise test. While the significance of a negative test has been established(1), "false positive" responses occur not infrequently in healthy individuals. Our experiment was based on the electrophysiologic principle that *duration* of RS-T depression in a given cycle is more important than *extent* of its depression. Thus, RS-T segment depression due to current of injury produced by subendocardial ischemia should persist for a greater portion of ventricular systole than the depression associated with simple tachycardia, or benign changes in ventricular gradient(2).

Material and method. 234 consecutive "2-Step" tests in which there was RS-T segment depression after exercise, were analyzed. In each case, the resting 12 lead tracing was entirely normal. There were 163 men and 71 women, all followed for 2 to 8 years, average 4 years. On the basis of comprehensive clinical evaluation and follow-up, these were divided in 2 groups: (a) 100 patients with definite organic ischemic heart disease (77 males and 23 females) and (b) 134 without organic heart disease (86 men and 48 women). Standard lead II and leads V_2 through V_6 were recorded in all patients immediately after exercise, as well as in 2 minutes and 6 minutes. Duration of RS-T segment depression in electrical systole, independent of heart rate, was measured according to the technic of Lepeschkin and Surawicz(3). In Fig. 1 the RS-T segment is deviated below the baseline. X is the point of return of depressed segment to isoelectricity. The time from beginning of ventricular activation to point X (QX interval) is expressed as percentile of QT (electrical systole) interval for that complex. (QX/QT ratio). The QX/QT ratio was recorded in every lead and in each time interval, wherever RS-T segment depression, however minimal, was present. Intervals were measured by calipers. In each case, the greatest QX/QT ratio was recorded, independent of the extent of RS-T depression in mm.

Results. The mean QX/QT ratio among 100 organic patients, both male and female, was 57.4%, exceeding 50% in all but one case.

The mean ratio among individuals without heart disease, was 43.9%, all but 12% failing to attain values of 50% or greater. These findings are illustrated in cumulative frequency distribution curves (Figs. 2a, 2b). Analysis by sex indicates that only 4 of 86 functional males had ratios of QX/QT greater than 50% (4.7%), whereas 12 of 48 women in the "no heart disease" category exceeded this value.

The overall diagnostic accuracy of QX/QT ratio when applied to evaluation of any RS-T depression after the "2-Step" test is 92.8%. The technic, however, is 97% accurate in males.

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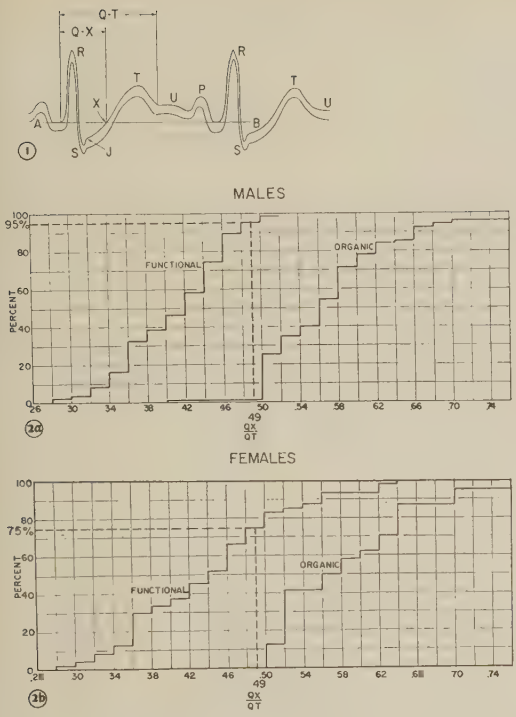

FIG. 1. Modified from Lepeschkin, E. and Surawicz, B., *New England J. Med.*, 258, 1958. RS-T segment is depressed. J is junction between end of QRS complex and beginning of RS-T segment. X is point of return of the depressed RS-T segment to isoelectricity. QX is time measured from beginning of ventricular activation to point X. QT interval (electrical systole)is measured from earliest deflection of QRS complex to end of T-wave. AB, the baseline or line of isoelectricity, is drawn through 2 successive QRS complexes.

FIG. 2a. Bar type cumulative frequency distribution curve in males indicates that 96% of individuals without heart disease have QX/QT ratios below 50%. With exception of one patient, all organic cases have values of 50% or more.

FIG. 2b. Cumulative frequency distribution bar graph of QX/QT ratio in women. All patients with organic heart disease have QX/QT ratios exceeding 50%. Among functional cases, 75% do not attain a 50% ratio.

There was no apparent relationship between QX/QT ratio and extent of RS-T depression in either organic or functional cases.

Conclusions. 1) There is a statistically valid difference in the nature of depressed RS-T segment after the "2-Step" test, among functional cases *vs.* those with ischemic heart disease. In presence of coronary heart disease, the depression persists for greater portion of ventricular complex than in functional cases. The QX/QT ratio is a simple and convenient expression of this characteristic of the RS-T segment and corrects for changes in heart rate. 2) A QX/QT ratio of 50% appears the optimal point at which to separate organic from functional cases. In 100 patients with known coronary heart disease, 99% demonstrate QX/QT ratios of 50% or more. Using this criterion, total incidence of false positive responses is 12% but with only 4.7% false reactors occurring among men. The overall accuracy of the criterion in both organic and functional cases with RS-T depression is 92.8% in our series. 3) It is suggested that criteria of an abnormal "2-Step" test should henceforth include a QX/QT ratio of 50% or more.

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Received November 5, 1959. P.S.E.B.M., 1960, v103.

Pantothenic Acid, Thiamine and Folic Acid Levels at Parturition.* (25504)

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Evidence showing fetal malformations and death from deficiencies of pantothenic acid (1), Vit. B_{12}(2), folic acid (PGA)(3), riboflavin(4), Vits. A, E, and K(5,6,7) have been

* This study was supported by U. S. Public Health Service Grants.

TABLE I. Pantothenic Acid, PGA and Thiamine Content of Maternal (M) and Infant (I) Serum at Parturition. All values given in mμg/ml.

Quartile	Pantothenic acid M	Pantothenic acid I	PGA M	PGA I	Thiamine M	Thiamine I
	×100					
1st	.5- 1	1- 5	1- 4	3.5- 10	3 -10	19- 55
2nd	1 - 2.5	5-15	4- 7	10 - 34	10 -11.5	
3rd	2.5-10	15-19	7- 14	34 -100	11.5-15.5	55-100
4th	10 -22	19-68	16-100	100 -250	15.5-30	
Median	2.5	15	7	34	11.5	55
No. of patients	51	64	42	26	49	10

described. Before one can attempt to correlate the effects of vitamins on fetal survival and development, normal ranges must be established for serum levels of the various vitamins throughout pregnancy and parturition. Vit. B_{12}, PGA, and riboflavin and various amino acid concentrations of maternal serum were found to be lower than infant's at parturition(8,9,10,11,12). This is attributed to the avidity of the fetus for these vitamins. In the present report, a comparison of pantothenic acid, thiamine and PGA serum levels of the normal mother and infant at parturition is made so as to establish standard values.

Methods. Assays for pantothenic acid, thiamine and PGA on maternal and infant serum were carried out by methods previously described(13,14,15). Maternal blood was collected from an antecubital vein and infant blood was taken from the cord at time of delivery. Specimens were obtained from patients followed by the Obstetrical Service of Mount Sinai Hospital. The patients were in good nutritional state.

Results are given in Table I. The median value of 250 mμg/ml of pantothenic acid in maternal serum is substantially lower than 1500 mμg/ml in infant serum. The same relationship holds for thiamine and PGA levels; 11.5 mμg/ml for thiamine in maternal serum versus 55 mμg/ml for infants; 7 mμg/ml compared to 34 mμg/ml of PGA for mother and infant serum respectively. Median vitamin levels in infant serum are approximately 5 × greater than the maternal. Only 19% of the infant values for pantothenic acid fell below the maternal median. Only 23% of the infant PGA levels fell below the maternal median.

In reference to the range of these vitamins in nonpregnant subjects(13,14,15), the pantothenic acid levels of mothers fall within the normal range (100-1,000 mμg/ml); that of infants is elevated. The mother's PGA level is depleted, compared to the normal range of 8-24 mμg/ml, while the infants' are higher than normal. Finally, thiamine is lowered in maternal circulation; the infant appears to be within normal range (25-80 mμg/ml).

The PGA results confirm previous findings (9). Maternal PGA and B_{12} levels are lowered during pregnancy(8,9,11), suggesting that circulating maternal B_{12} and PGA levels are depleted by the fetus for use in cellular synthesis, a process catalyzed by these vitamines(9,16).

The maternal values for thiamine at parturition are lower than those reported for nonpregnant normals(14). In cases of pregnancy complicated by beriberi, thiamine deficiency was implicated as a factor responsible for toxemias(17). The maternal thiamine levels reported here (Table I), suggest that thiamine reserve is limited and that further reduction of maternal thiamine, as in nutritional deficiency, could be one of the factors leading to toxemias of pregnancy.

Summary. A comparison of pantothenic acid, thiamine, and PGA serum levels of mothers and infants at parturition shows an approximately 5-fold increase of these vitamins in the fetal circulation. The significance of these findings is discussed.

We are indebted to Dr. Alan F. Guttmacher, Dept. of Obstetrics and Gynecology, and his nursing staff for their cooperation.

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Received November 9, 1959. P.S.E.B.M., 1960, v103.

Plasma Protein VI. Catabolism *in vitro*.* (25505)

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It is apparent from numerous studies on turnover that serum albumin is being synthesized and degraded at a rapid rate in the mammal. The data of Cohen and coworkers (1) indicate that degradation occurs in the rabbit at 48 mg/hour. The present work was designed to determine the site of breakdown in the rat by carrying out *in vitro* studies with tissue slices and homogenates incubated with albumin which had been randomly labeled with C^{14}. Studies of this nature have already been carried out by Roberts and Kelley(2), who labeled rat albumin by administering phenylalanine-3-C^{14} to donor rats and then measured catabolism of labeled albumin in tissue slices from livers of the same species. They reported that albumin was rapidly broken down, a result that we have been unable to repeat. Katz and coworkers (unpublished) have likewise been unable to confirm results of Roberts and Kelley, using iodinated and S^{35}-labeled serum albumin. Other workers investigated the breakdown of albumin in liver either *in vivo* or by using perfusion technic (with or without reticuloendothelial blockade with india ink). Results of such experiments are not in good accord. Miller and coworkers (3) reported considerable breakdown of plasma protein by the perfused rat liver, whereas Gordon(4) found that perfused rat liver broke down albumin at a rate of only 10 to 15% of that calculated for the intact animal from turnover data. However, it has been reported that partial hepatectomy in rats (5) and mice(6) results in decrease in turnover rate of albumin. Following reticuloendothelial blockade various workers have reported conflicting results(6,7,8).

Methods. Slices or homogenates of rat tissues were incubated at 37° in buffer with plasma protein or albumin randomly labeled with C^{14}, made by feeding donor animals with labeled *Rhodospirillum rubrum*(9,10). Serum albumin was separated by salt fractionation. The method used was based on observations of Butler and Montgomery(11), and involved precipitation of globulins with 4 vol. 2.4 *M* phosphate buffer at pH 6.5. The solution was allowed to stand at room temperature for 24

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TABLE I. Serum Albumin Breakdown *In Vitro.*

System	Tissue used, mg	Labeled substrate,* mg	SA† $\times 10^{-3}$	Incubation time, hr	Activity $BaCO_3$‡	Recorded TCA soluble, %
Rat liver slices						
Bicarb.§	300	A (15)	4	3.5	None	None
Phosph.‖	100, 160	A (10)	4	2, 2.5	"	"
Rat tissue homogenate						
Liver-bicarb.	200–750¶	P (1-15)	2.4	3.5–5	"	"
"	600	A (9)	4	5	"	"
Kidney-phosph.	500	A (10)	4	2.5	"	"
Spleen- "	600	A (10)	4	2.5	"	6.2
Ascites- " **	500–600	A (10)	4	2.5–5.5	"	None
Liver- "	700	AH (5)	(21)††	2.5	+	–30‡‡

* A, serum albumin (randomly labeled with C^{14} from *Rhodospirillum rubrum* grown in $C^{14}O_2$ containing medium); P, total plasma protein labeled as A; AH, acid hydrolysate of A (5 mg).

† SA, specific activity in terms of cpm/mg protein.

‡ Activity recorded from CO_2 in ionization chamber, generally from 60 mg of $BaCO_3$. When standard samples of $C^{14}O_2$ (100 dpm) were passed through chamber, activity registered was very much above background, showing that apparatus was functioning at normal efficiency.

§ Bicarbonate buffer at pH 7.4 (Cohen(13)).

‖ Krebs-Ringer phosphate buffer at pH 7.4 (Cohen(13)).

¶ 5 incubation experiments.

** Ehrlich ascites tumor cells from C^3H mouse; wt of cells estimated from packed cell vol.

†† Total activity added to incubation mixture (cpm).

‡‡ Since all activity added was TCA-soluble, activity disappeared from medium due to catabolism of amino acids to $C^{14}O_2$.

hr. Then the albumin solution was filtered off, subjected to thorough dialysis and lyophilized. Its purity was evaluated by electrophoresis on paper. Total plasma protein in some experiments was obtained by thorough dialysis of C^{14}-labeled blood plasma. Radioactivity in solid samples was determined by sedimenting samples on aluminum planchets followed by counting with G.M. counter in conventional manner. However, to increase sensitivity, CO_2 produced from labeled albumin or other substrate was introduced into the chamber of a vibrating reed electrometer and its activity measured by rate of charge method(12). By this method the sensitivity for detecting radioactivity was increased by a factor of 5 above that observed with a G.M. tube with a thin window. Activity is reported as cmp or dpm (disintegrations/minute), depending on whether the G.M. counter or Electrometer was used in determination of radioactivity. Tissue slices or homogenates were incubated under conditions given in the Table, in the presence of 1% CO_2 in 99% oxygen. When tissue slices were used, they were added to 2 ml of labeled albumin solution made up in either bicarbonate or phosphate buffer (see footnotes, Table I). Tissue homogenates were made up to contain the amount of tissue used in 2 ml of buffer. To this was added the albumin dissolved in 2 ml of the same buffer. Two to 4 liters of gas mixture were passed slowly through the incubation flask, and into a pair of traps containing NaOH solution. In all experiments a water trap (conc. H_2SO_4) and ionization chamber (250 ml) were placed between incubation flask and NaOH traps, to monitor the activity directly. However, in no case, except with the hydrolysate of albumin, was sufficient radioactivity present to make rate of charge differ significantly from background rate. When the period of incubation was over, sulfuric acid was tipped into the incubation mixture to drive out any residual CO_2. The CO_2 trapped in the NaOH solution was precipitated as $BaCO_3$. CO_2, generated from weighed amounts of the precipitate, was introduced into the evacuated ionization chamber for determination of its activity. Contents of the flask used for incubation, were treated with sufficient trichloroacetic acid (TCA) to precipitate all the protein. After centrifuging the protein, aliquots of the supernatant were dried on polyethylene planchets, and activity was determined with a G.M. counter. The difference between activity found after incuba-

tion and that found at zero time is recorded in last column of Table. The amount, expressed as per cent of activity added, is taken to represent the fraction of the protein broken down to amino acids and soluble peptides.

Results of these experiments which were nearly all negative are reported in Table I. There was no significant production of $C^{14}O_2$ from labeled albumin or plasma protein, although when albumin was hydrolyzed, some of the amino acid carbon was catabolized to $C^{14}O_2$ (last line of Table). The only significant breakdown of labeled albumin to give TCA soluble products was found with spleen homogenate incubated at pH 7.4.

Gordon(4) reported that perfused liver from rats, degraded albumin at 1 mg/hour. Hence, assuming analogous behavior of liver slices, 160 mg of liver slices in 2.5 hours should have degraded 27 μg of albumin, or 108 cpm should have been released. In the ionization chamber this 108 cpm is equivalent to approximately 540 dpm. One-tenth of this activity should have easily been detected. Therefore, it is necessary to conclude, either that albumin does not penetrate the tissue slice with sufficient rapidity so that breakdown is demonstrable in 2.5 hours, or that breakdown of albumin depends on its concentration, and that this was not sufficiently high in these experiments. However, this should hardly have been the case in experiments involving homogenates. If the first situation prevails it is not clear why albumin was not broken down in the homogenates, unless breakdown requires some type of structural integrity.

It is difficult to explain why our results are in such great contrast with those of Roberts and Kelley(2). Even though a different type of labeling was employed by these workers, it is not apparent why an albumin labeled with phenylalanine-tyrosine-3-C^{14} should behave differently from albumin labeled in any other way *provided the albumin has not become modified in some way during its preparation.* It might be suggested that phenylalanine-tyrosine-3-C^{14} is reincorporated into newly synthesized albumin in liver slices to a greater extent than are the randomly labeled C^{14}-amino acids. Such a phenomenon has been shown to occur in the intact rabbit by Penn and coworkers(14). However, if reincorporation of essential amino acids does occur to a considerable extent in tissue slice experiments, the question arises as to why methionine-cystine-S^{35} was not reincorporated in the investigations of Katz and Sellers(3). Thus all that remains is to suggest that at least part of the albumin used by Roberts and Kelley had suffered a denaturation, rendering it more vulnerable to breakdown.

In view of the lack of breakdown of albumin in our work, and the low rate of breakdown observed in liver perfusion experiments of Gordon(4), as compared with the high rate anticipated from turnover data, we are investigating other possible sites of serum albumin catabolism.

Summary. 1. Rat serum albumin randomly labeled with C^{14} amino acids was prepared. 2. Neither this serum albumin, nor dialyzed whole plasma similarly labeled, was broken down to any significant extent by any of the tissue slice or homogenate systems tested, although it was anticipated that this should have been demonstrable from breakdown rates calculated from turnover data and from liver perfusion data. 3. It is suggested that either serum albumin breakdown depends on its concentration, the concentration being too low in our experiments; or that albumin is broken down at some other site in the animal.

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Received November 9, 1959. P.S.E.B.M., 1960, v103.

Passive Transfer of Resistance to Tuberculosis Through Use of Monocytes.* (25506)

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Numerous attempts have been made to elucidate the mechanism of acquired resistance to tuberculosis. In particular, the possible role of cellular changes in acquired resistance has received considerable attention. Initial experiments by Lurie(1) indicated that rate of multiplication of tubercle bacilli was reduced in monocytes obtained from immunized animals when compared to monocytes obtained from nonimmunized animals. These early experiments were performed with a technic which employed the anterior chamber of eyes of nonimmunized rabbits as culture medium. Rich(2) however, using tissue culture technics, was unable to demonstrate inhibition of multiplication of tubercle bacilli in leukocytes obtained from immunized animals. Recent experiments utilizing improved tissue culture methods also resulted in divergent findings. Suter(3) reported significant inhibition of multiplication of tubercle bacilli in monocytes obtained from immunized guinea pigs when compared to multiplication in monocytes obtained from normal guinea pigs. He also found that serum obtained from immunized animals had no influence on results. Mackaness(4) on the other hand, reported no difference in this respect between monocytes from normal and immunized rabbits. Fong *et al.* (5) indicated that effective resistance to virulent tubercle bacilli by immune monocytes required continuous presence of immune serum. Lastly, Berthrong and Hamilton(6) recently reported that monocytes from immune guinea pigs limited intracellular multiplication of virulent tubercle bacilli. A direct resolution of the role of cells in acquired resistance to tuberculosis would involve the demonstration of transfer of resistance to tuberculosis to normal animals through use of monocytes from immunized animals. The present paper deals with experiments demonstrating this transfer.

Materials and methods. Mice of Strong A strain were used. Animals weighing initially 18-22 g were housed in separate metal cages in groups of 10 and were fed food and water *ad lib.* Mice were challenged by administration of 1 mg wet weight of H37Rv strain of *Mycobacterium tuberculosis* var. *hominis,* For this purpose, 3 week old surface pellicle cultures were harvested from modified Proskauer and Beck medium and standardized, using methods previously described(7). Immunized animals employed as donors for passive transfer experiments were given 1 mg wet weight of BCG‡ intravenously 3 weeks before sacrifice. These mice were immunized at appropriate consecutive intervals so that in each instance of passive transfer, donor preparations were available of the same age and as nearly alike as possible. A summary of ma-

* Supported in part by a research grant from the Tuberculosis Institute of Chicago and Cook County.

‡ BCG strain 4 was furnished through courtesy of Dr. Sol Roy Rosenthal, Tice Clinic, Univ. of Ill., Chicago.

TABLE I. Effect of Passive Transfer of Material from Normal Mice on Survival of Normal Mice.

	Material transferred	No. of mice	Median survival time in days and 95% confidence limits
1.	Plasma	35	14.8 (12-17)
	None	30	14.6 (12-17)
2.	Macrophages	25	16.2 (14-18)
	None	30	14.8 (12-17)
3.	Plasma & macrophages	50	16.0 (14-18)
	None	45	15.2 (13-18)
4.	Spleen homogenate	35	15.2 (14-18)
	None	30	15.0 (13-18)
5.	Spleen filtrate	20	15.6 (14-18)
	None	20	15.2 (14-18)

terials transferred and number of animals employed is given in Table I and II. Materials were obtained from either normal or BCG immunized mice and in each instance, were transferred the day before and 2 and 5 days after challenge by intravenous administration of 0.4 ml of appropriate preparation. All donor animals were sacrificed by hyperextension of the neck. Blood was obtained by thoracotomy and opening the beating heart. Blood from sacrificed mice was pooled, and serum removed for administration. Macrophages were obtained from peritoneal cavity by stimulation with 1 ml Klearol† given 5 days before transfer. Immediately after sacrificing, peritoneal cavities were opened and in each instance washed with 5 ml of chilled Tyrode's solution. Pooled washings were centrifuged 5 minutes at 250 g in refrigerated centrifuge (4°C). The supernatant was discarded and cells resuspended and centrifuged in chilled Tyrode's solution for additional 5 minutes at 250 g in refrigerated centrifuge. The supernatant was again discarded and the cells suspended in sufficient chilled Tyrode's solution to give a concentration of 400,000 to 600,000 monocytes/0.2 ml. Number of white cells in blood and peritoneal exudate was determined by counting in hemocytometer. Differential white cell counts were made from smears of blood and peritoneal exudates stained with Wright's stain. Spleen preparations were made by homogenization of spleens using size "A" Teflon grinders and matching ignition tubes. For each spleen, 5 ml of chilled Tyrode's solution was added. After 10 minutes homogenization, the preparation was diluted to contain 100 mg tissue in 0.2 ml. Equivalent filtrates were prepared from similar preparations by passing homogenized material through large pore sintered glass filter. In no instance did more than 2 hours elapse between time of sacrifice of donor animals and injection of cells, serum, or tissue into recipient animals. All materials transferred were given by intravenous route. The following preparations were employed in transfers: 1. For serum: 0.2 ml serum + 0.2 ml Tyrode's sol. 2. For monocytes: 0.2 ml monocytes in Tyrode's sol. + 0.2 ml Tyrode's sol. 3. For serum and monocytes: 0.2 ml serum + 0.2 ml monocytes in Tyrode's sol. 4. For spleen homogenate: 0.2 ml of homogenate + 0.2 ml of Tyrode's sol. 5. For spleen filtrate: 0.2 ml of filtrate + 0.2 ml of Tyrode's sol. The methods of Youmans and Youmans(8) were used for producing standardized tuberculous infection in mice and for evaluation of immune response.

Results. Table I gives results with material transferred from normal mice, while Table II gives results with material transferred from BCG immunized mice. Separate controls were employed in each experiment. Control animals were selected at random and received no materials at time passive transfers were made with test animals. Analysis of mouse white

TABLE II. Effect of Passive Transfer of Materials from BCG Immunized Mice on Survival of Normal Mice.

	Material transferred	No. of mice	Median survival time in days and 95% confidence limits
1.	Plasma	65	15.0 (13-17)
	None	60	14.8 (12-17)
2.	Macrophages	25	18.8 (17-21)
	None	20	14.6 (12-17)*
3.	Plasma & macrophages	70	19.2 (17-21)
	None	65	14.2 (12-16)†
4.	Spleen homogenate	35	14.8 (12-17)
	None	30	14.6 (13-17)
5.	Spleen filtrate	30	15.0 (13-17)
	None	20	15.2 (13-17)

* Significant prolongation of median survival time ($P = 0.05$).

† *Idem* ($P = 0.01$).

† Klearol, a highly refined mineral oil.

blood cell counts and differential counts revealed that the number of white cells was between 3,000 and 5,000 cells/ml^3, both before and after passive transfer. Distribution of types of cells was: Polys 10-18%; Lymphocytes 64-73%; Monocytes 6-11%; and Eosinophiles 0.2-0.5%. No white cell determinations were made after fifth day of challenge.

A comparison of white blood cell determinations before and after passive transfers indicated that no significant change occurred in total number of white cells after transfers. However, after each of the first 2 transfers of monocytes, the differential white cell count showed elevation of monocyte level to 12-18% of total count. This elevated level fell to near normal within 3 days after each of the first 2 passive transfers. No determinations of this type were made after third transfer.

Passive transfer experiments conducted with various preparations indicate that transfers involving macrophages from BCG immunized mice resulted in significant prolongation of survival. Prolongation of survival was small but significant at a level at which $P = 0.05$. Simultaneous transfer of macrophages and plasma from immunized mice resulted in prolongation of survival of recipient animals, statistically signifiacnt at a level at which $P = 0.01$. No other materials from immunized mice nor any materials from normal mice affected survival time.

Discussion. Passive transfer of resistance to tuberculosis through use of monocytes from immunized animals has been demonstrated by our experiments. In no instance was the effect as great as that seen with active immunization with BCG(8), nor did any of the other materials tested have any effect on survival. Simultaneous transfer of monocytes and plasma from immunized animals further demonstrated the transfer of resistance to tuberculosis, however the effect was not significantly different from that found with monocytes alone.

Transfer of monocytes resulted in 50-100% increase in peripheral monocyte level each time they were given. This is in agreement with the theoretical level expected in animals of this size. If it is assumed also that all transferred cells were viable and became fixed in tissues, each animal received a total of approximately 1.5 million additional functioning tissue monocytes. Since the infecting dose of tubercle bacilli contained approximately 40,000,000 viable particles(9) there would be one monocyte transferred for every 26-27 tubercle bacilli or clumps of tubercle bacilli. This number of tubercle bacilli/monocyte is well within the limits observed capable of being ingested by monocytes *in vitro*(3-6). However, normal monocytes of recipient animals would be expected to engulf many infecting organisms, thus making the effect of transferred cells less pronounced.

A similar increase in monocyte level has been noted in animals injected with BCG(10) and in human beings with active tuberculosis. Based on these observations it appears likely that a greater prolongation of survival would occur with transfer of larger numbers of monocytes from immunized animals.

While the existence of active cellular mechanisms resulting in acquired resistance to tuberculosis, appears to be established, the degree to which these mechanisms function in the infected animal remains to be elucidated. The question raised by investigations of Sever and Youmans(9,10) as to whether a quantitative change in monocyte response or a qualitative change in monocytes is involved in acquired resistance to tuberculosis, would appear to be partially resolved. Since passive transfer of monocytes obtained from normal animals produced no response while use of the same number of cells obtained from immunized animals increased resistance, it appears that a qualitative change in monocytes themselves was necessary. It is still possible that a larger number of normal monocytes than was used, might also produce some increase in survival time of mice.

The nature of the qualitative change in monocytes remains to be established. Some mechanisms available to the altered monocytes have been suggested by investigations of Sever and Youmans(9,10). Based on their observations, it appears that acquired resistance to tuberculosis may be due, at least in part, to rapid migration of macrophages into tuberculous areas which would result in local anoxia sufficient to inhibit the tuberculous

process. Also, Howard *et al.*(11) observed that increased metabolic activity of cells of the reticulo-endothelial system of BCG immunized animals may bring about development of intracellular environment unfavorable for bacterial multiplication. Should these cellular alterations function to their fullest extent, availability of immune monocytes in localized areas of infection could be sufficient to confine and inhibit the tuberculous process.

Summary. Experiments are reported which demonstrate passive transfer of a limited degree of increased resistance to tuberculosis through use of monocytes from immunized animals. Use of monocytes from normal animals had no effect on resistance. No increased resistance was seen in animals receiving plasma, spleen homogenate, or spleen filtrate obtained from either normal or immunized animals.

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Received November 9, 1959. P.S.E.B.M., 1960, v103.

Chromatography of Proteins of Squamous Cell Carcinomas and Normal Epithelium of Mice.* (25507)

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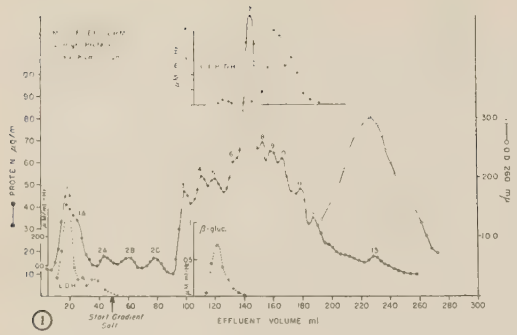
Electrophoresis has been used to fractionate proteins of extracts of various tumors and normal tissues(1,2). The method of chromatography of proteins on substituted cellulose ion exchange columns has been used in this laboratory to fractionate soluble proteins of liver and several enzyme activities localized in various fractions(3). The present paper is concerned with chromatographic fractionation of proteins of extracts from squamous cell carcinomas and normal epidermis of mice. Three enzymes have been localized in the fractions.

Methods. The normal epidermis of mice was removed from dermis mechanically as reported previously(4). Tumors used were: (a) primary squamous cell carcinomas induced in Swiss mice by painting the skin with methylcholanthrene; and (b) 3 lines of transplanted squamous cell carcinomas carried for several generations in this laboratory. Extracts were prepared by homogenizing at 0°C at high speed with a "Virtis" homogenizer in .005 *M* tris (hydroxymethyl)aminomethane-phosphate buffer, pH 8.00 ± 0.03. The homogenates were then spun 30 to 45 minutes at 13,500 rpm in Sorvall SS-1 centrifuge; the clear supernatants were dialyzed overnight against the same buffer, then stored frozen at −15°C. Chromatography on diethylaminoethylcellulose anion-exchange columns was carried out as reported previously(3). These methods will be reported later. Proteins were determined by the Lowry method(5); in addition absorbances at 280, 260 and 413 mμ were measured in each fraction. For β-glucuronidase assay, the Fishman method was used(6); for lactic-dehydrogenase (LDH),

* This work was supported by Grants from U.S.P.H.S. and Am. Cancer Soc.



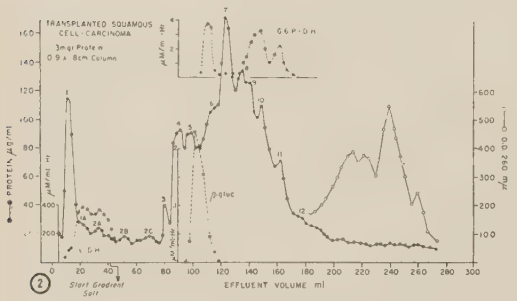


FIG. 1. Typical chromatogram of extract of normal epidermis on DEAE-SF.

FIG. 2. Typical chromatogram of extract of squamous cell carcinomas on DEAE-SF.

the Kornberg method(7); and for glucose-6-phosphate dehydrogenase (G-6-PDH), the Buell, *et al.*, method was used(8). Six experiments with extracts of normal epidermis, and 4 with extracts of each type of tumor were carried out.

Results. In all experiments the protein patterns showed some similarity (Fig. 1,2), the various peaks appearing approximately at the same chloride concentrations during chromatography of either normal or cancer tissue. The material in peaks 1 through 12 showed typical protein ultraviolet absorption, while that appearing between 0.3 and 0.5 *M* NaCl had maximum absorbance at 260 mμ. Hemoglobin, evidenced by 413 mμ absorbing material, appeared in all chromatograms in 2 or more fused peaks between 0.05 and 0.07 *M* NaCl. Nucleic acids (260 mμ absorbing material) appeared in only one sharp peak in all chromatograms of normal epidermis extracts; in chromatograms of all tumor preparations they appeared in a wider zone with 2 major peaks (Fig. 1,2). Whether this is related to a different quantitative or qualitative composition of nucleic acids in the extracts of tumors and normal epidermis requires further investigation. The graphs also show distributions of 3 enzyme activities; other chromatograms gave the same patterns with some variations in relative peak heights. LDH activity in chromatograms of normal epidermis appeared as one sharp peak corresponding to the first protein peak, followed by at least 2 smaller peaks. In all tumor chromatograms, LDH activity was present in a wider zone localized in a region following first protein peak and was shifted to the right as compared with its position in normal chromatograms (Fig. 2).

β-glucuronidase activity was localized in the same place in all chromatograms, with one sharp peak which was higher in extracts of tumors than in those of normal epidermis. Glucose-6-phosphate dehydrogenase activity showed 3 distinct peaks in tumor chromatograms (Fig. 2): however, in the chromatograms of extracts of normal epidermis there was evidence of only the 2 more tightly bound peaks, the first one extremely low or absent. Appearance of this first high peak of activity in tumor preparations can be correlated with the reported observation of increase of glucose-6-phosphate dehydrogenase activity in neoplastic liver homogenates(9).

Localization of these 3 enzymes in the chromatograms of tumors as well as in normal epi-

TABLE I. Ratio between Chloride Concentration Required to Elute the Various Peaks of Enzyme Activities and Chloride Concentration Required to Elute the Nucleic Acid Peak.

		(Cl^-) enzyme peak/(Cl^-) N.A. peak			
		G-6-PDH			
Tissue	No. exp.	Peak 1	Peak 2	Peak 3	β-glucuronidase
Liver	3	.20	.40	.48	.15
Epidermis	5		.41	"	"
Transplanted tumors	9	"	.40	"	"
Induced tumors	4	"	"	.47	"

dermis was comparable to that found during chromatography of mouse liver extract by the same procedure. When the positions of various peaks of activity were expressed relative to chloride concentration required to elute the major peak of nucleic acids (Table I), the β-glucuronidase activity appeared in each chromatogram in the same relative position.

In liver extracts glucose-6-phosphate dehydrogenase activity appeared as 3 peaks (Table I). Each peak of activity of this enzyme in tumor and epithelium chromatograms corresponded to one of the 3 of liver.

Summary. These results show that the above chromatographic procedure can be used to separate different protein extracts into a number of fractions and to investigate the distribution of various enzyme activities to pick out qualitative enzymic differences between malignant tumors and the normal tissues from which they arise.

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Received November 12, 1959. P.S.E.B.M., 1960, v103.

Free Phenolic Substances in Blood Plasma.*† (25508)

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Veldhuis(1), Nakao and Aizawa(2), Aitken and Preedy(3), Slaunwhite and Sandberg(4), and Oertel *et al.*(5) have described chemical methods for determination of estrogens in blood. The methods of Nakao and Aizawa, and Aitken and Preedy employ column chromatography for final separation of the estrogens prior to estimation by fluorometry. In our laboratory an alumina column similar to that described by Nakao and Aizawa was employed. However, instead of eluting the column with 1% ethanol in benzene for the estrone fraction, elution was carried out with 0.25, 0.50 and then 1.0% ethanol in benzene. When this was done, it was found that the free "estrone" fraction actually consisted of a substance slightly less polar than estrone. This phenolic substance less polar than estrone would be estimated as estrone if elution were performed with only 1.0% ethanol in benzene. The following is a description of results of estimations of free estrone and estradiol-17β fractions in plasma. Some properties of a substance less polar than estrone are also described.

Method. Heparinized blood was centrifuged immediately after withdrawal and the plasma separated. The method of Brown (6) was adapted for extraction. Plasma (20 ml) was extracted by partition once with 4 volumes of ether for 30 min. and then twice with 2 volumes for another 15 min. The ether extract was washed with 16 ml of concentrated sodium carbonate at pH 10.5 and partitioned with 4 ml of 2 N sodium hydroxide. The sodium hydroxide was adjusted to pH 10.5 by addition of 16 ml of 8% sodium bicarbonate,

* This work supported by U. S. Public Health Grant and John G. Clark Memorial Fund. Technical assistance of Roberta Messick is gratefully acknowledged.

† The authors are indebted to Drs. Britton Chance and Lester Packer, Johnson Foundation, Univ. of Pennsylvania for micro absorption spectrometry.

shaken with the ether, and the aqueous phase discarded. The ether was successively washed with 4 ml of 8% sodium bicarbonate, and twice with 2 ml of water and evaporated to dryness. An alumina column that gave consistent separation and recovery was developed. The alumina column described by Stimmel (7) and more recently by Nakao and Aizawa (2) was modified using Harshaw alumina "catalyst grade" which was deactivated by the addition of 6 ml of water per 100 g of alumina. One end of a glass tube (0.5 x 30 cm) was plugged with glass wool and filled with benzene. Two grams of alumina were allowed to settle through the benzene. The alumina was then washed with 12 ml of benzene and the extract transferred to the column with 32 ml of benzene in 4 ml aliquots. This was followed by 12 ml of 0.25% ethanol in benzene. The estrone was eluted by 24 ml of 0.50% ethanol in benzene, and the estradiol-17β with both 1% and 5% ethanol in benzene. Four ml fractions were collected. The solvents were evaporated in a vacuum oven at a temperature of 55-60°C. For fluorometry 4 ml of 88% sulfuric acid was added to each fraction, agitated thoroughly and allowed to stand for 30 minutes. The acid solution was then transferred to Farrand tubes (10 x 70 mm), heated in an oven for 6 minutes at 100°C and allowed to cool for 20 minutes to room temperature. The fluorescence was determined in a Farrand Model A photofluorometer with a primary filter of maximum transmission at 436 mμ and a secondary filter with maximum transmission of 480 mμ. Spectrophotofluorometric studies have shown that these wavelengths were optimal for the estrogens under discussion, when sulfuric acid solutions were used.

Results obtained with a plasma extract separated by the alumina column using 1% ethanol in benzene for elution of estrone, compared with an equivalent plasma separated with the 0.25 and 0.50% ethanol in benzene eluting mixtures showed that there appeared a substance less polar than estrone with the latter procedure. A peak in the estradiol-17β fraction, and a peak between this and the "estrone" were found. Free estrone was not always present in plasma extracts separated by

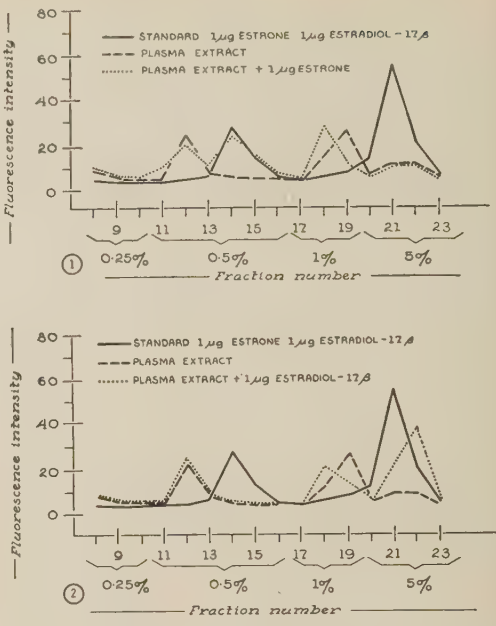

FIG. 1. Behavior of 1 μg of estrone added to an extract before chromatography.

FIG. 2. Behavior of 1 μg of estradiol-17β added to an extract before chromatography.

the column. In order to determine whether the peak less polar than estrone was an artefact caused by the extraction procedure, estrone and estradiol-17β were added to individual plasma before and after extraction, prior to chromatography. After chromatography the material less polar than estrone was always present. The added estrone and estradiol-17β appeared in the same fraction as did the estrogens of the reference column. The same estrogens extracted from aqueous solution did not show the substance less polar than estrone after chromatography. Fig. 1 shows the results of chromatography of a plasma extract to which estrone had been added. Fig. 2 gives the result after addition of estradiol-17β. No appreciable peaks were found in a solvent blank.

In later work phenolic separation between toluene and 1 N sodium hydroxide as described by Engel(8) prior to chromatography on alumina was done. The substance less polar than estrone was phenolic. The material between estrone and estradiol-17β was not always present. The fraction corresponding to estradiol-17β was not affected by the phenolic separation and is phenolic.

The absorption spectrum of the substance less polar than estrone when dissolved in sulfuric acid showed maxima at 400 and 420 mμ which are not shown by any estrogen available to us. The fluorescence of this compound in sulfuric acid was found to be maximal at 480 mμ with maximal excitation at 440 mμ which is characteristic of the estrogens.

Studies with the fraction corresponding to estradiol-17β have shown that the fraction may contain phenols other than estradiol-17β. The absorption spectrum of the fraction in sulfuric acid showed maxima at 420 and 490 mμ with an inflection at 455 mμ which estradiol-17β does not show. In sulfuric acid fluorescence properties were the same as those of estradiol-17β. However, this fraction when studied for estrogenic potency produced a positive vaginal smear in castrate female mice.

The amounts of estrone in 2 of 15 blood samples from pregnant women were 7.6 and 2.3 μg per 100 ml of plasma. The amounts of the fraction corresponding to estradiol-17β in the 15 samples averaged 6.5 μg/100 ml blood, calculated as estradiol-17β. It must be emphasized that this figure includes the increment that is not estradiol-17β.

These results agree with the preliminary report of Purdy, Engel and Oncley(9) that estrone is not found in free form in blood. They showed that estrone sulfate was the main metabolite found in blood after administration of estradiol-17β. The nature of the unknown materials is being investigated.

Summary. A detailed examination of the free estrone fraction of alumina column chromatograms of extracts of blood plasma has shown that when elution is performed with 0.25, 0.50 and then 1% ethanol in benzene instead of only using 1% ethanol in benzene, the free "estrone" fraction actually consisted of a substance slightly less polar than estrone. Free "estrone" is seldom found in plasma. Two of 15 samples contained 7.6 and 2.3 μg/100 ml plasma. The fraction which presumably contained estradiol-17β contained other material. The so-called estradiol-17β fraction averaged 6.5 μg/100 ml plasma.

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Received November 12, 1959. P.S.E.B.M., 1960, v103.

Factors Affecting Drug Metabolism by Liver Microsomes. IV. Starvation.* (25509)

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We have studied effects on drug metabolism of factors which affect liver structures, especially liver microsomes(1). One such factor is starvation, since electron micrographs of liver tissue showed that differences do exist between normal and fasted animals. Cytoplasmic basophilia decreases sharply after starvation of a few days, and most hepatic cells lose ergastoplasm(2) and undergo significant change in dispersion of this structure(3). We believed that these cellular changes in liver structure: namely, reduction in amount of ergastoplasm, and changes in microsome subunits, might have an effect on certain microsomal drug metabolisms. A variety of drugs are metabolized through the action of en-

* This investigation supported in part by U.S.P.H.S. Grant.

TABLE I. Effects of Starvation on Drug Metabolism by 9000 × g Supernate from Pooled Mouse Liver. (Micromoles drug metabolized†/g of liver.)

	Incubation time (hr)	Normal	Starved animals			
		S.T.* 5-15 min.	S.T.* 20-40 min.	S.T.* 41-60 min.	S.T.* 61-80 min.	S.T.* greater than 80 min.
Hexobarbital	1½	4.46 (4) [2.40–5.96]	2.79 (4) [1.86–4.00]	1.86 (4) [.84–2.34]	.88 (2) [.56–1.20]	.77 (4) [.32–1.11]
Chlorpromazine	"	2.80 (3) [2.49–3.33]	1.98 (3) [1.27–2.38]	1.70 (3) [1.26–2.22]	1.37 (3) [1.21–1.56]	1.19 (3) [.98–1.36]
Pyramidon	"	1.48 (4) [1.42–1.53]	1.11 (4) [.74–1.25]	.74 (4) [.60–.80]	.57 (4) [.35–.70]	.46 (4) [.36–.57]
Acetanilid	"	2.03 (4) [1.30–2.50]	2.22 (4) [1.27–3.42]	1.68 (4) [1.00–2.71]	1.21 (3) [.96–1.68]	.93 (3) [.86–1.03]
p-Nitrobenzoic acid	1	8.22 (3) [5.76–12.30]	17.03 (3) [15.72–18.78]	14.62 (3) [14.16–15.12]	11.88 (3) [11.49–12.60]	8.43 (3) [7.76–8.92]
Neoprontosil	1	15.55 (3) [12.06–19.38]	17.65 (4) [12.39–20.55]	16.44 (3) [10.50–20.91]	15.37 (2) [11.37–19.38]	12.59 (3) [9.90–14.85]

* Groups made on basis of hexobarbital sleeping times (S.T.). See *Methods.*

† Amount of drug disappeared in the case of hexobarbital or chlorpromazine, amount of product formed in case of pyramidon, acetanilid, p-nitrobenzoic acid, or neoprontosil. These values are avg of several experiments. Figures in parentheses are No. of experiments made, and figures in brackets are ranges obtained in the determinations.

zymes present in liver microsomes(4). A greater sensitivity to drugs of the fasted animal may be in part due to deficiency of these enzymes. To gain a picture of the effects of starvation, various drug pathways were studied. These included side chain oxidation of hexobarbital, N-dealkylation of pyramidon, hydroxylation of the aromatic ring of acetanilid, oxidation of the ring sulfur of chlorpromazine, reduction of aromatic nitro-group of p-nitrobenzoic acid, and reduction of aromatic azo-group of neoprontosil.

Methods. Male mice of initial weight of 25-30 g were used. After 36 hours starvation, hexobarbital (80 mg/kg administered intraperitoneally) sleeping times were determined, and mice grouped as to those which slept 20-40, 41-60, 61-80, and more than 80 minutes. Normally, control mice given the same dose of hexobarbital slept less than 10 minutes. Any starved mice sleeping less than 19 minutes were considered essentially unaffected by starvation; this group never amounted to more than 15-20% of total animals used. Twelve hours later (total starvation of 48 hours) metabolism *in vitro* of various drugs by pooled livers from groups of mice was determined. Supernate fraction (9000 g), containing microsomal and soluble enzymes, was prepared from the homogenate of pooled liver samples by high speed angle centrifuge. Methods used in measuring drug metabolism are the same as in the first paper of this series (1 a). Incubation times were the same for liver samples from normal and starved animals.

Results. Table I shows that *in vitro* metabolism correlated well with prolongation of hexobarbital sleeping times. Thus, animals which slept longest were also those whose livers were least able to metabolize most drugs studied. Exceptions are pathways involved in reduction of aromatic nitro group or azo linkage. *In vitro* metabolism of compounds like p-nitrobenzoic acid and neoprontosil is not depressed by starvation which causes a marked effect on most oxidative pathways. Whether or not starvation results in a true stimulation of reduction as compared with marked depression of oxidation is now being studied.

We have also shown that the metabolism of drugs in these fasted animals returns completely to normal within 24 hours after access to food. Under investigation is the effect of various diets on return to normal, of ability of animal to metabolize drugs.

We determined that the dose of hexobarbital used in grouping starved mice, *i.e.,* in assessing effects of starvation on metabolism *in vivo,* does not result in differences in drug enzyme activity, when animals so treated are

compared with uninjected controls. Thus, drug metabolisms in livers from animals injected 12 hours previously with a dose of 80 mg/kg of hexobarbital were compared with those in livers from uninjected animals and shown to differ by no more than 10%. Therefore, when we assayed metabolisms *in vitro*, previous drug administration had had a minimal effect. Such experiments were prompted by the work of Conney *et al.*(5) who showed drug administration to stimulate certain microsomal drug metabolisms measured 24 hours to several days after such drug administration.

Apparent lack of enzyme activity in livers from starved animals could be due to (I) actual absence of enzyme protein; (II) deficiency of cofactors, *i.e.*, reduced triphosphopyridine nucleotide (TPNH); or (III) presence of inhibitors of drug metabolizing enzymes.

We tried to eliminate the second possibility by adding TPNH-generating system of glucose-6-phosphate, glucose-6-phosphate dehydrogenase, triphosphopyridine nucleotide (TPN),† and nicotinamide.

Presence in starved liver of inhibitors of drug metabolism was not suggested in experiments where metabolism by starved and normal livers determined separately was compared with metabolism by mixtures of starved and normal livers.

We are left with the probability that the decrease in enzyme activity is due to an actual loss in enzyme protein.

Discussion. Electron micrographs of samples of liver from starved animals reveal changes in structure of the endoplasmic reticulum from which microsomes are derived. Such structural changes may be associated with marked depression of drug metabolism which we have shown to occur in starved animals as compared with normal animals. The nutritional status of the animal therefore becomes important as a possible reason for some of the so-called biological variation in drug response. Animals may vary in ability to metabolize drugs on the basis of whether or not they have had sufficient food prior to drug administration. A properly controlled experiment should include regulation of food intake before any drug administration.

Our findings may have possible therapeutic importance in administration of drugs to severely debilitated and/or malnourished patients. Our finding that depressed drug metabolisms recover fairly rapidly upon refeeding is encouraging in this connection. However, it should be pointed out that a state of starvation reached quickly may not be fully comparable with a similar state reached slowly. Animals maintained on insufficient diets for extended periods of time may not respond as quickly upon refeeding, in recovering normal levels of drug metabolism, as the experimental animals used.

Summary. Starvation depresses hepatic microsomal drug metabolism both as measured *in vitro* and *in vivo*. This depression is believed to be caused by an actual loss of enzyme protein in the microsome. Oxidative pathways are affected more than reductive pathways and indeed starvation may result in an activation of reduction of nitro and azo groups. The nutritional status of the animal prior to drug administration becomes important as a factor in the response obtained. Possible therapeutic implications are discussed.

† Chemicals obtained from Sigma Chemical Co., St. Louis, Mo.

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Received November 12, 1959. P.S.E.B.M., 1960, v103.

Succinylcholine-Induced Contractures in Skeletal Muscles of Newborn Cat.* (25510)

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It has long been known that during the first postnatal weeks mammalian skeletal muscles (1,2), like those of denervated muscles(3) have an increased sensitivity to acetylcholine. This ACh-sensitivity has recently been demonstrated with more refined technics and the results so obtained indicate that the choline ester depolarizes immature(4) and denervated(5) muscle fibers over their whole surface. Since denervation is the only known factor clearly associated with ACh-sensitivity of mature mammalian muscles, the question arises as to whether this similar property of immature muscles is related to their incomplete functional innervation. Opportunity for testing this possibility was afforded during preparation of newborn kittens for neurophysiological studies, in which it was noted that intravenous succinylcholine produced generalized contractures. Since choline ester-induced contractures have been demonstrated in skeletal muscles of the mature mammal only after denervation(6,7), attempts were made to establish the relationship between succinylcholine-contractures and functional innervation in the newborn kitten.

Methods. Over 45 kittens, ranging in age from late fetal stage to 3 weeks, were initially anesthetized with ether to permit introduction of a tracheal and external jugular vein cannula, then allowed to recover after local anesthetization of exposed skin margins. Concentric needle electrodes were inserted into limb muscles for oscilloscopic registration of their electrical activity. The animals were tested for various types of reflex responses after which succinylcholine chloride (0.2-5 mg/kg in 0.5-1 ml Ringer's) was injected and artificial ventilation instituted.

* Financed by the NINDB and the Paul Moore Fund.

† Scholar, Kenny Fn.

‡ Visiting Fellow in Neuropharmacology, NIH.

Results. Skeletal muscles of unanesthetized, intact kittens, less than 2 days old, participated vigorously in a variety of excitatory and inhibitory reflex activities. Muscle action potentials from leg extensors elicited by minimal, but sustained stretch were rapidly eliminated during and for some time after pressure to the ipislateral foot pad (Fig. 1A). Contralateral foot pad pressure markedly augmented leg extensor activity, whereas superimposed ipislateral foot pad pressure abolished it (Fig. 1B). These findings do not support the conclusion that spinal inhibitory reflexes are poorly developed in 1-2 day old cats(8). On the contrary, they indicate existence of complex powerful spinal reflex pathways operating in the intact animal by the second postnatal day.

In the absence of spontaneous or evoked movements, skeletal muscles were electrically silent. Under such conditions, succinylcholine evoked a flurry of action potentials. Electrical silence (Fig. 1C) again ensued during development of generalized contractures of such severity as to rapidly induce a postural attitude which superficially resembled that seen in the decerebrate, mature cat (Fig. 2). This posture resulted from sustained contractures in both flexors and extensors; activity of the latter group predominating in neck, trunk and hindlimbs, the former, in the distal and to a lesser extent, proximal forelimb musculature.

Duration and intensity of the generalized contractures depended on age of animal and interval between successive injections of succinylcholine. Contractures, best demonstrated in late fetal and neonatal animals (less than 3 days), were less prominent in 3-7 day old kittens and absent after 10th-12th postnatal day. By the end of second week, succinylcholine produced effects entirely similar to those observed in mature animals, *i.e.*, gen-

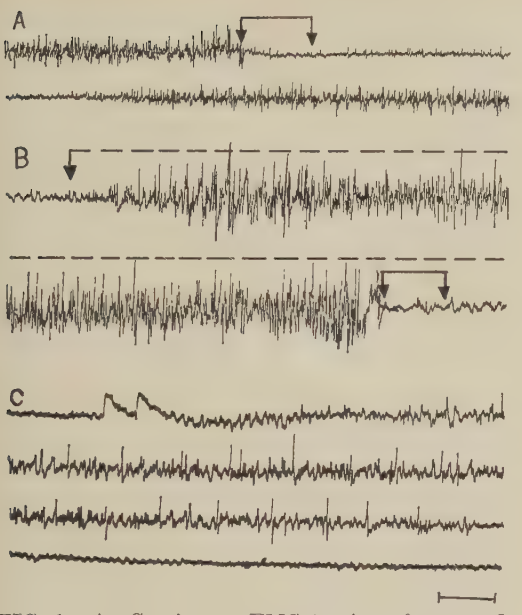

FIG. 1. A. Continuous EMG tracings from quadriceps under light stretch in 2-day-old intact kitten. Arrows indicate approximate duration of pressure to ipislateral footpad. Note persisting inhibition of quadriceps activity despite maintained stretch. B. Same preparation as in A, quadriceps relaxed. At arrow, pressure applied to contralateral foot pad evokes crossed extensor reflex activity (broken line) which is promptly abolished by ipislateral foot pad pressure (solid line). C. Continuous EMG tracing from relaxed quadriceps of another 2-day-old animal. Tracing commences a few seconds after intrav. succinylcholine (2 mg/kg). This induced an outburst of muscle action potentials which terminated in electrical silence. Time cal. 4 sec. in A; 2 sec. in B, and 1 sec. in C.

eralized twitchings and rapid flaccid paralysis. In the perinatal group, contractures, as judged by manipulation of limbs and head, were of extraordinary duration (3-10 min) and intensity. When these subsided, there generally supervened a prolonged period of flaccid paralysis (1-2 hours), after which spontaneous movements might return. Subsequent injections of succinylcholine then produced transient contractures or none at all prior to paralysis. Frequently, spontaneous movements were not observed for 3-4 hours after severe contractures induced by concentrations of succinylcholine 2-3 times higher than that routinely employed in this laboratory to paralyze adult cats (1-2 mg/kg).

Comment. The effects of intravenous succinylcholine on skeletal muscles in the newborn cat resemble that produced by close-arterial injections of relatively high concentrations of ACh in chronically denervated muscles of the mature animal(6,7). A large ACh-induced contracture in denervated muscle leaves in its wake a profound depression of excitability to electrical or subsequent chemical stimulation(6,9). In the newborn cat, succinylcholine-contractures appear to induce a similar depression as indicated by absence of spontaneous movements for long periods and failure of successive injections of succinylcholine to evoke contractures despite the fact that the choline ester may block neuromuscular transmission when some recovery does occur. Our study provides no direct information on the mechanism by which succinylcholine produces contracture in newborn cats. During ontogenesis(4), muscle fibers, like those in chronically denervated muscle (5), are sensitive to the depolarizing action of

FIG. 2. Overt effects of succinylcholine in 1½-day-old kitten. *Above,* kitten, relaxed, lying on right side during recovery from ether anesthesia. *Below,* postural attitude 1 min. after intrav. succinylcholine (4 mg/kg). Note opisthotonus, extension of hindlimbs and flexion of distal forelimbs. Generalized rigidity persisted for 5 min. before flaccid paralysis supervened. Spontaneous limb movements were not observed even after 3 hr.

ACh over their whole surface and this sensitivity is associated with a high myosin-ChE and low myosin-ATP'ase activity(10,11). Global chemosensitivity of muscle fibers in the newborn animal could satisfactorily account for contracture-inducing effects of succinylcholine reported here. There is, however, no satisfactory explanation as to why succinylcholine fails to evoke contractures in the cat after 10th-12th postnatal day. If this is related to the finding that at this time the chemosensitivity of muscle fibers is restricted to the end-plate region(4), then it must be allowed that the changing responsiveness of skeletal muscles in newborn cats to succinylcholine is not *immediately* related to establishment of motor innervation, but to some factors which are brought into operation during the latter phases of ontogenesis.

Summary. Intravenous succinylcholine produces generalized, prolonged contractures in newborn cats prior to 10th-12th postnatal day. This sensitivity of immature muscles to succinylcholine is not related to detectable deficits in their functional innervation.

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Received November 13, 1959. P.S.E.B.M., 1960, v103.

Polymyxin B and Colistin: Activity, Resistance and Crossresistance *in vitro*.* (25511)

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Polymyxin B, a polypeptide antibiotic available for over a decade, has been used successfully in treatment of serious Gram-negative bacillary infections resistant to other antibacterial agents. It has been particularly effective against infections due to *Pseudomonas aeruginosa* but totally ineffective against Proteus infections; its use, however, has been limited by its neuro- and nephrotoxicity(1). Colistin, a new antibiotic recently introduced in Japan as Colimycin, is also a polypeptide and is produced by *Bacillus colistinus,* which is related to *B. polymyxa,* the source of polymyxin; it has similar antibacterial action but apparently lacks much of the toxic properties of polymyxin B. Both antibiotics have similar chemical constituents; each contains (+)-6-methyl-octanoic acid, d-leucine, l-leucine, l-threonine and 1-α,γ-diaminobutyric acid but polymyxin B contains d-phenylalanine which colistin lacks and the numbers of some of the other constituents differ(2-6). This paper deals with comparisons of *in vitro* activity of these 2 antibiotics against pathogenic organisms, most of them Gram-negative bacteria recently isolated from patients, and also reports observations on development of resistance and crossresistance to these 2 antibiotics *in vitro*.

Materials and methods. Polymyxin B sul-

* Aided by grant from Nat. Insts. of Health.

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TABLE I. Susceptibility of Pathogenic Bacteria to Polymyxin B (P) and Colistin (C) *In Vitro.*

Organism	No. of strains*	Range of M.I.C., μg/ml P	Range of M.I.C., μg/ml C	Comparison of M.I.C.'s P = C %	P > C %	P > C Avg P/C	C > P %	C > P Avg C/P
Escherichia coli	26	.8- 3.1	.4- 3.1	42	23	1.8	35	1.6
Aerobacter	24†	.8->100	1.6->100	38	29	2.0	33	2.0
Klebsiella pneumoniae	13	.8- 100‡	.4- 100‡	38	38	1.5	23	1.8
Pseudomonas aeruginosa	29	3.1- 12.5	3.1- 12.5	17	0		83	1.8
Shigella	40	.8- 3.1	.4- 3.1	53	20	1.9	28	1.5
Salmonella	32	.8- 3.1	.8- 3.1	47	16	1.9	37	2.0
Neisseria gonorrhoeae	25	25 - 100	25 ->100	8	8	2.0	84	3.4
Hemophilus influenzae	43	.4- 3.1	.8- 25	5	5	1.6	91	4.0
Staphylococcus aureus	26	≧100§	>100					
All except *S. aureus*	232			30	15		55	

M.I.C. = Minimum inhibiting concentration, calculated as base.

* Only strains tested simultaneously with both P and C are included.

† 13 were *A. aerogenes* (M.I.C. 1.6-6.3) and 11 were *A. cloacae*; 3 of the latter were not inhibited by 100 μg/ml of P or C and 8 were susceptible to ≤25 μg/ml.

‡ Only 1 strain was inhibited by 100 μg/ml of P and C; the rest were inhibited by ≤6.3 and ≤3.1 μg/ml of P and C, respectively.

§ Only 1 strain was inhibited by 100 μg/ml.

fate (Aerosporin) was supplied by Burroughs Wellcome & Co.; colistin was supplied either as sulfate (Coly-mycin S) or as methanesulfonate (Coly-mycin M) by Warner Lambert Research Inst. but only the sulfate was used in the *in vitro* studies. All concentrations are expressed in terms of weight equivalent of base. Strains of most species tested for sensitivity to these antibiotics were recently isolated from blood, urine or infected exudates submitted for culture to the Bacteriology Laboratory, but most strains of *Escherichia coli* and *Klebsiella pneumoniae* were of specific types obtained from Dr. P. R. Edwards, Communicable Diseases Center, Chamblee, Ga., who also furnished strains of *Aerobacter cloacae and A. aerogenes*. Sensitivity of strains to the antibiotics was determined after growth for 24 hours on plates of heart infusion agar, Difco, pH 7.2±, into which 2-fold dilutions of the antibiotics were incorporated, the highest concentration tested being 100 μg/ml. "Chocolate" agar was used for strains of *Hemophilus influenzae* and *Neisseria gonorrhoeae,* and incubated under increased CO_2 (in candle jar) for 48 hours. Minimum inhibiting concentration (M.I.C.) was considered the lowest concentration with no perceptible growth or very slight growth visible only with a magnifying lens (3×); in comparing activity of the 2 antibiotics, the latter type of growth was arbitrarily rated as 50%, whereas moderate inhibition (+ growth, cf +++ growth on antibiotic-free medium) was rated as 75% of the 2-fold increment in M.I.C.

Results. Susceptibility of various organisms to Polymyxin B and Colistin. Results of tests for sensitivity of 258 strains of 9 genera or species of bacteria to the 2 antibiotics are summarized in Table I. All 26 strains of *S. aureus* were resistant to 100 μg/ml of both antibiotics, except one that was inhibited by this concentration of polymyxin. Several strains of various streptococci and other Gram-positive cocci as well as strains of Proteus (not listed in Table) were tested and found resistant to 100 μg/ml, except a strain of *S. lutea* which was inhibited by 50 μg/ml of polymyxin and 100 μg/ml of colistin. Of 232 strains of Gram-negative organisms listed in Table I, 30% were equally susceptible to both antibiotics, 55% were more susceptible to polymyxin and only 15% were more sensitive to colistin. The differences were usually only slight, and for many individual strains were less than one 2-fold dilution, *i.e.,* one antibiotic produced complete inhibition in the same minimum concentration that produced partial inhibition by the other. Average ratios of inhibiting concentrations are listed in Table I for strains of each genus or species in which the M.I.C. of the 2 drugs differed. Average ratio was usually less than 2, except for *H.*

TABLE II. Resistance and Crossresistance after Repeated Subcultures on Agar Containing Polymyxin B or Colistin.

	Antibiotic used for test	*Pseudomonas aeruginosa* (mucoid, green)	*Pseudomonas aeruginosa* (brown)	*Aerobacter aerogenes*	*Escherichia coli*
		μg/ml			
Parent strain:					
M.I.C.	P	12.5	3.1	1.6	1.6
	C	12.5	3.1	3.1	3.1
G.E.C.	P	3.1	.4	.4	.4
	C	3.1	.8	.8	1.6
After 30 subcultures on P:					
M.I.C.*	P	>100 (10)	6.3	>100 (5)	3.1
	C	" "	6.3	" (5)	3.1
G.E.C.*	P	" (20)	.8	" (8)	.8
	C	" "	.8	" (10)	.8
After 30 subcultures on C:					
M.I.C.*	P	" (10)	1.6	(5)	1.6
	C	" "	6.3	" (5)	3.1
G.E.C.*	P	" (20)	.8	" (10)	.8
	C	" (17)	.8	" (6)	.8
After 30 subcultures on antibiotic-free agar:					
M.I.C.	P	3.1	6.3	1.6	1.6
	C	3.1	3.1	3.1	1.6
G.E.C.	P	.4	.4	.4	.4
	C	.8	.8	.8	.4

Abbreviations: P = polymyxin B; C = colistin; M.I.C. = minimum complete inhibiting concentration; G.E.C. = maximum concentration on which growth is equal to that of control culture on antibiotic-free agar.

* No. in parentheses indicates the No. of subcultures required to achieve this value (crossresistance tests done only after each fifth subculture on homologous antibiotic).

influenzae against which polymyxin was, on the average, 4 times as active as colistin for 91% of the strains, and among those of *N. gonorrhoeae,* for 84%, of which the M.I.C. of colistin was, on the average, 3.4 times greater than that of polymyxin.

Development of resistance and crossresistance in vitro. Two studies were carried out to produce resistant strains, one by multiple steps and the other by a single step. Four culturally and biochemically distinct strains originally susceptible to both polymyxin and colistin were used. In the first experiment, each strain was subcultured on 2 series of agar plates, one containing serial dilutions of polymyxin and the other similar dilutions of colistin, as in tests for sensitivity. After incubation for 48 hr at 37°C, 2 mm loopful of the growth was scraped from surface of plate from each series containing maximum concentration of antibiotic on which growth was equal to that on control plate of antibiotic-free agar (G.E.C.); this was suspended in 1 ml of broth and the suspension used to inoculate another similar series of plates containing the same antibiotic. After each 5 subcultures on the same antibiotic, the inoculum was also used simultaneously in test for sensitivity to the heterologous antibiotic. The M.I.C. and G.E.C. were recorded at each subculture and a total of 30 transfers on each antibiotic were thus carried out, the parent strains also being transferred on antibiotic-free agar each time. Changes in susceptibility to homologous and heterologous antibiotics are summarized in Table II. High degrees of resistance to each drug with complete crossresistance developed in the green strain of *Ps. aeruginosa* and in the strain of *A. aerogenes,* but the susceptibility of the brown Pseudomonas and the *E. coli* did not change significantly.

In the second experiment, the same 4 strains were used to derive single-step mutants by growing a large inoculum on the surface of antibiotic-containing agar. Sedimented organisms from 10 ml of a 24 hr growth in

TABLE III. Resistance and Crossresistance to Polymyxin B and Colistin in Single-Step Mutants.

Organism	Inoculum*	Antibiotic in agar†	No. of colonies		M.I.C., μg/ml	
			At 48 hr	Tested	Polymyxin B	Colistin
Ps. aeruginosa (green)	10	P 100	0			
	10	C 100	5	2	12.5, 3.1	6.3, 12.5
	25	P 200	0			
	"	C 200	0			
	"	P 1000	0			
	"	C 1000	0			
Ps. aeruginosa (brown)	10	P 100	0			
	"	C 100	6	3	3.1, 6.3, 1.6	1.6, 1.6, .8
	25	P 200	5	2	3.1, 3.1	25, 25
	"	C 200	3	2	.4, .4	1.6, .8
	"	P 1000	1	1	no growth	no growth
	"	C 1000	2	2	"	"
A. aerogenes	10	P 100	>100	2	>100, >100	>100, >100
	"	C 100	?			
	25	P 200	5‡	2	" "	" "
	"	C 200	>100	2	" "	" "
	"	P 1000	> 50?	2	no growth	no growth
	"	C 1000	0			
E. coli	10	P 100	0			
	"	C 100	0			
	25	P 200	0			
	"	C 200	0			
	"	P 1000	0			
	"	C 1000	0			

* ml of culture, the centrifuged sediment of which was used as inoculum for each plate.
† P = polymyxin B, C = colistin; No. indicates concentration in μg/ml.
‡ Also >50 tiny colonies that failed to grow on subculture with or without antibiotic.

brain-heart infusion broth were resuspended in about 1 ml and flooded over the surface of 2 agar plates, one containing 100 μg/ml of polymyxin and the other a similar amount of colistin. The plates were allowed to stand with porous clay covers for several hours at room temperature until the surfaces were nearly dry, then incubated at 37°C with the porous covers until the surfaces were completely dry when glass covers were replaced and incubation was continued for 36 to 48 hr. After that time, the number of colonies that developed were noted and representative ones were picked, emulsified in small amount of broth and the suspension streaked on the surface of antibiotic-free agar and on agar containing 12.5 μg/ml of the homologous antibiotic to bring out any dependent variants. All organisms that grew on the antibiotic-containing agar also grew in absence of antibiotic, indicating that none of them were antibiotic-dependent. Growth from the antibiotic-free agar was then subcultured to broth and the resulting growth used to test for sensitivity to both antibiotics. The same procedure was repeated using the sediment of 25 ml of culture on agar containing 200 and 1000 μg/ml of each antibiotic.

The results are summarized in Table III. A few sensitive "persistors" were identified in growth of green Pseudomonas on 100 μg/ml of colistin and in growth of brown Pseudomonas in concentrations of 100 or 200 μg/ml of one or both antibiotics. One-step, highly resistant mutants, however, developed only in *A. aerogenes* on 100 or 200 μg/ml of either antibiotic. Dependent variants could not be demonstrated.

Comment. Our data indicate that colistin is comparable with polymyxin B in its activity *in vitro* against strains of organisms that are susceptible to the latter. Previous observations by Jawetz and Coleman (7) indicated that high degrees of resistance to polymyxin B could be obtained by daily subcultures using large inocula of *A. aerogenes* or *Ps. aeruginosa* that were originally of intermediate susceptibility to that antibiotic, but only a

slight rise in resistance could be achieved with 14 daily transfers of an originally highly susceptible strain of *E. coli*. Wright *et al.*(8) subjected 6 strains of *E. coli* to 20 subcultures in presence of polymyxin B and demonstrated increased resistance (12.5 to >400 μg/ml) in only one of them. Two of 4 strains showed marked increases in resistance by repeated subculture in either polymyxin or colistin and this was accompanied by complete cross-resistance to the heterologous drug. One of the same 2 organisms also yielded one-step highly resistant mutants, which also showed complete crossresistance.

Summary and conclusions. Polymyxin B and colistin, 2 closely related polypeptide antibiotics, had similar antibacterial activity against a large number of pathogenic bacteria. Polymyxin was somewhat more active than colistin against 55% of susceptible strains, on the average 3.4 and 4 times as active against most strains of *N. gonorrhoeae* and *H. influenzae*, respectively. Highly resistant variants developed on repeated subculture of a strain of *A. aerogenes* and one of *Ps. aeruginosa* on agar containing either of these antibiotics; complete crossresistance to the other antibiotic developed in each instance. Single-step mutants of the same strain of *A. aerogenes*, highly resistant to each antibiotic, were readily obtained by seeding a large inoculum on the surface of agar containing 100 or 200 μg/ml of that antibiotic; these mutants were completely cross-resistant to the heterologous antibiotic. Antibiotic-dependent variants were not encountered.

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Received November 16, 1959. P.S.E.B.M., 1960, v103.

Experimental Histoplasmosis in Monkeys.* (25512)

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Previous studies from this laboratory have been concerned with pathogenesis of histoplasmosis in mice(1-4), dogs(5), and other large animals(6). The present report compares infectivity of *Histoplasma capsulatum* for *Macaca mulatta* when administered by intratracheal (IT), intranasal (IN), intragastric (IG) and intravenous (IV) routes.

Materials and methods. Young adult monkeys were inoculated by various routes above with 5 ml of saline suspension of yeast phase *H. capsulatum*† containing 2 ml of packed cells from 4-day cultures grown on brain heart infusion (Difco) blood agar. Eight monkeys were inoculated IT(7), 4 IN(8), 3 IG(9) and 2 IV (long saphenous). Blood cultures, taken 4, 7, 14, and 25 days after inoculation when possible, and all tissues taken at autopsy were treated and cultured by the method of Conant (10). The serologic response of monkeys was followed by collodion agglutination(11) and complement fixation(12) tests. Periorbital subcutaneous tests(13) for sensitivity to histoplasmin were carried out before inoculation and on surviving animals 6-7 weeks after inoculation.

Results. Table I summarizes the findings in 17 monkeys inoculated with *H. capsulatum* by various routes. All 8 monkeys inoculated IT exhibited clinical evidence of infection.

* Supported by Nat. Inst. Health grant.

† Strain G13—an isolate from spontaneous histoplasmosis in a dog, originally obtained from Charlotte Campbell Army Medical Center, Washington, D.C.

TABLE I. Response of Monkeys to Viable Yeast Phase *H. capsulatum* Given by Various Routes.

Monkey No.	Route	Symptoms*	Blood culture, day pos.	Histoplasmin skin test	Antibody response	Disposition: Died, days	Disposition: Sacrificed, mo	Histoplasma isolated at autopsy
1	Intratracheal	G-acute	ND†	ND	—	7		Liver, spleen, mesenteric, bronchial and axillary nodes. Placenta but not fetus.
2		GR-acute	—	+	+		4	None
3		G-acute	ND	ND	—	7		Lung, spleen, bronchial node. Placenta but not fetus.
4		GR-acute	—	+	+		4	None
5		"	—	+	+		6	"
6		GR-mild	4, 7	+	—		4	"
7		"	—	+	+		24	"
8		"	—	+	—		4	Bronchial node
9	Intranasal	C	—	+	—		4	*Idem*
10		GR-mild	7	+	+		24	None
11		C	—	+	—		4	Bronchial node
12		C	—	+	+		6	None
13	Intragastric	TD	—	—	—		4	"
14		"	—	—	—		4	"
15		"	—	+	—		4	"
16	Intravenous	G-acute	4	ND	—	8		Lung, liver, spleen, mesenteric & bronchial nodes.
17		"	7	ND	—	10		*Idem*

* G = Generalized symptoms—fever, weight loss, anorexia, lethargy, abnormal coat. R = Respiratory symptoms—cough, dyspnea, trachypnea, wheezing. C = Cough only. TD = Transient diarrhea.
† ND = Not done.

Two pregnant monkeys (Nos. 1,3) in this group died 7 days after inoculation. Positive cultures were obtained at necropsy from both animals, from various tissues including placentae but not the fetuses. Of the remaining 6 monkeys, 1 (No. 8) of 4 sacrificed at 4 months yielded a positive culture (bronchial node); 1 (No. 6) yielded positive blood cultures at 4 and 7 days, and 2 (Nos. 2,4) exhibited serologic and skin test evidence of infection. Monkeys 5 and 7 sacrificed after 6 and 24 months, respectively, showed skin test and serologic evidence of infection, but cultures were negative.

A mild generalized infection was observed in 1 (No. 10) of 4 monkeys inoculated IN, while the other 3 exhibited cough only (Table I). Positive blood cultures were obtained on 7th day in No. 10 which subsequently showed a positive skin test and serologic evidence of infection; when sacrificed at 24 months, no positive cultures were obtained. Two monkeys (Nos. 9,11) developed positive skin tests but no serologic evidence of infection; however, when sacrificed at 4 months both yielded positive cultures from the bronchial nodes. The 4th monkey in this group (No. 12) developed positive skin test and circulating antibodies but had negative cultures when sacrificed at 6 months.

The 3 monkeys inoculated IG showed no clinical evidence of infection although a mild transient diarrhea was observed during first 24 hours. All blood cultures were negative, and none exhibited demonstrable antibodies. One monkey developed a positive skin test. No positive cultures were obtained when sacrificed at 4 months.

Both monkeys inoculated IV died 8 and 10 days later after an acute fulminating disease process. Positive blood cultures were obtained from both animals ante-mortem, and *H. capsulatum* was isolated from various organs at autopsy. Neither demonstrated anti-

bodies during this short period.

Discussion. The above results indicate that monkeys (*M. mulatta*) are susceptible to infection by yeast phase *H. capsulatum,* administered intravenously, intratracheally and intranasally but not by the gastric route. The clinical course of the illness, appearance of organisms in the blood stream, development of sensitivity to histoplasmin, recovery of organisms at autopsy, and especially the character and extent of antibody response, point to the fact that infection was indeed established. DeMonbreun(14) produced infection in 2 rhesus monkeys inoculated intravenously with yeast phase organisms. Hill and Marcus(15) however, were unable to infect monkeys (*M. irus*) with *H. capsulatum.* These workers inoculated 6 monkeys intratracheally with yeast phase cells in mucin suspension and noticed no gross evidence of infection. Skin tests remained negative, and no antibodies were produced. Intracardial inoculation in 5 monkeys also failed to produce obvious infection, although 3 of 5 became sensitive to histoplasmin and all developed antibodies. Differences in results of various studies are undoubtedly referable to either differences in the strain of *H. capsulatum* used, size and nature of inoculum, or monkey species employed.

In the present study, 2 monkeys inoculated intratracheally died of fulminating disease 7 days after inoculation. The fact that both were pregnant females may have had some bearing on the severity of infection. *H. capsulatum* was recovered from various organs of both animals and from the placentae, but not from either fetus. The organisms were apparently unable to pass the placental barrier. These observations are of interest as they relate to histoplasmosis in pregnant humans.

Our study supports the belief that the respiratory tract is the most common, or likely portal of entry of the organism in spontaneous histoplasmosis. The failure to infect monkeys by introducing the inoculum directly into the stomach seems to minimize the importance of water or other ingested material in pathogenesis of this infection.

Summary. Severe disease was produced in monkeys by yeast phase *H. capsulatum* administered intravenously and intratracheally. Monkeys inoculated intranasally exhibited a more mild disease process. No significant clinical signs of illness were seen in monkeys inoculated by the gastric route.

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Received November 16, 1959. P.S.E.B.M., 1960, v103.

Cytotoxic Action of Normal Human Serum on Ehrlich Ascites and Sarcoma 180 Cells.* (25513)

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Our recent studies(1,2) demonstrated markedly cytotoxic action of normal human serum on HeLa cells and spontaneously transformed U12 cells of human origin, serially propagated in tissue culture. No such effect was demonstrable on newly isolated or untransformed cell strains derived from normal tissue. Similar cytotoxicity of human serum was observed on atypical mouse cells, strain L and the DeBruyn Lymphosarcoma. Fedoroff(3,4) described in detail the effects of certain human sera on strain L, particularly that of schizophrenics. Recently Willheim *et al.* (5,6) have shown that normal human serum is able to digest Ehrlich ascites tumor cells, Sarcoma 180 cells, Krebs ascites tumor cells, and Walker rat ascites tumor cells. These observations demonstrate the presence of naturally occurring cytolysins or cytotoxins, whose presence may be related to natural tumor immunity. Further investigations were undertaken to determine if the cytotoxic effect of human serum on Ehrlich ascites tumor and Sarcoma 180 cells was similar to its action on strains HeLa, U12 and the other atypical human cell strains studied(2).

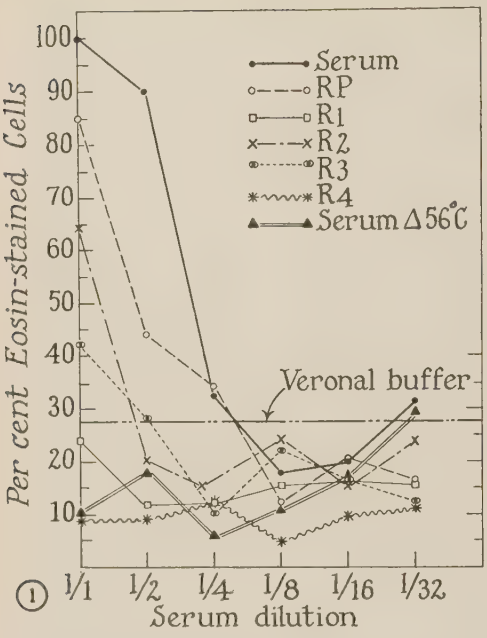
Materials and methods. Ehrlich ascites tumor† (EA) and Sarcoma 180‡ (Sa 180) were perpetuated by serial intraperitoneal injections of Strain CF-1 mice. Ascitic fluid was aseptically aspirated 10 days after injection. Only bloodless fluid was used. The cells were washed by serial centrifugation and suspended in synthetic cell medium S-15(1). Cell suspensions so prepared were incubated at 37°C for 1 hour with doubling saline dilutions of serum or serum reagents. After incubation, cytotoxicity was determined by Eosin staining method(1,7,8). Degree of cytotoxicity is the per cent of eosin-stained cells counted on a hemocytometer. Equivalent numbers of cells of both strains were incubated 1 hour with serum and heated serum. After washing, equal numbers of each pretreated strain were inoculated intraperitoneally into CF-1 mice to compare their ability to produce ascites tumors. Sera used were pools prepared from 25 to 50 donors, aliquots of which were frozen at –40°C and thawed when needed. In general R1, R2, R3, and R4 were prepared according to the method of Ecker *et al.*(9), the R4 prepared with NH_4OH. RP and RPb were prepared fresh for each experiment according to the method of Pillemer *et al.*(9). Serum was inactivated by heating at 56°C 1 hour (Serum Δ). Complement-fixed (CF) serum, deficient in C′1, C′2 and C′4, was prepared by precipitating in the serum, pneumococcal capsular polysaccharide type III with its type specific rabbit antiserum. Equal volumes of serum were adsorbed with doubling numbers of EA and Sa 180 cells to determine if toxic effects could be removed in this way. Aliquots of the EA and Sa 180 adsorbed sera were then tested on viable EA and Sa 180 cells respectively, using Eosin staining technic. Hemolytic titrations for the 4 components of complement were also performed(10) on aliquots of adsorbed serum, to determine if adsorption out of cytotoxicity was associated with inactivation of any of the components of C′.

Results. Toxicity of pooled human serum on both EA and Sa 180 cells is well shown by Eosin staining method. Allowing for variation in activity from one serum pool to another, susceptibility to injury by both cell strains is of approximately the same order; 1 to 5 x 10^6 cells killed/ml of serum. This is approximately 10 times the toxicity for strain U12 previously studied. These effects are

* This work supported in part by U.S.P.H.S. funds.

† EA tumor obtained from Atomic Energy Research Project, Western Reserve Univ. School of Med.

‡ Sa 180 obtained from Sloan-Kettering Inst. by Dr. Austin Weisberger.



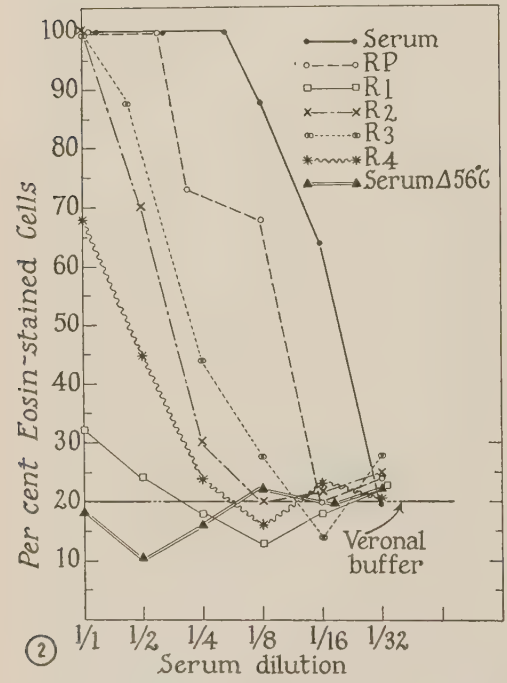


FIG. 1. Cytotoxicity of normal human serum and serum reagents of C′ on EA cells. 4.5×10^5 EA cells/ml of serum, serum reagents, or dilutions thereof, were used. Chart shows lack of cytotoxicity in Serum △, R1, R3, and R4. RP and R2 more closely approximate activity of native serum.

FIG. 2. Cytotoxicity of normal human serum and serum reagents of C′ on Sa 180 cells. 3.6×10^5 Sa 180 cells/ml of serum, serum reagent, or dilutions thereof, were used. Chart shows lack of toxicity mainly in Serum △ and R1. Toxicity of R4 is somewhat greater, while RP, R2, and R3 more closely approximate activity of native serum.

largely abolished by heating or complement-fixation of the serum.

With EA cells, the activity of R1, R3, and R4 is much less than native serum. The activity of RP and R2 is almost that of native serum. Addition of purified human properdin§ (0-30 u/ml) does not enhance activity of RP or RPb. Sa 180 cells are least affected by R1, while R2, R3, R4 and RP were closer to activity of native serum. Some of these observations are summarized in Fig. 1 and 2.

Treatment of EA and Sa 180 cells with serum prior to intraperitoneal inoculation in mice markedly inhibits formation of ascites tumor. 14-21 days following injection, mice so treated develop at most only a minimal accumulation of a parvicellular massive ascites within 1 week (Table I, Fig. 3).

Adsorption of serum with large numbers of cells produces a marked diminution in cytotoxicity for the adsorbing cell strain. Loss of serum toxicity by adsorption with EA and Sa 180 cells was not associated with significant inactivation of any of the components of C′. Results of a typical experiment are embodied in Table II.

Discussion. Heat-labile cytotoxins are present in normal human serum for Ehrlich ascites tumor and Sarcoma 180 cells, similar in some respects to cytotoxins active against human strains U12 and HeLa. A unit volume of serum kills approximately 10 times more EA and Sa 180 cells than U12 cells. Strains Sa 180, EA, U12 and HeLa show a similar ability to detoxify human serum by adsorption.

In all these situations, serum activity bears some relationship to serum complement, since heating and complement-fixation of serum eliminates the toxicity for all cells studied. Behavior of EA cells is more like strain U12 and HeLa in its reactions with the R′ reagents of C′, than Sa 180. Sa 180 deviates strikingly from the pattern, with only R1 being significantly less toxic than native serum. Adsorption of serum cytotoxicity with EA and Sa

§ Purified human properdin obtained from Dr. I. H. Lepow.

TABLE I. Inhibition of Ascites Tumor Formation by Sa 180 and EA Cells Pretreated with Human Serum.

		No. mice	Ascites tumor formation 0	+	++	+++	% ascites takes
Sa 180	Serum	16	16	0	0	0	0
	" △	16	0	2	3	11	100
EA	Serum	19	16	3	0	0	18.75
	" △	12	3	0	0	9	75

180 cells is not associated with significant inactivation of any of the components of C′, whereas with strain U12, this was associated with inactivation of C′2 and particularly C′4 (2). In most respects our observations on EA cells are in close agreement with those of Willheim *et al.*(5,6). The major exception is that these workers report marked loss of activity in RP, and hence conclude that properdin may be of importance in the reaction. Our data do not support this contention, since RP and RPb were only slightly less active than native serum and addition of properdin to these reagents did not enhance their activity on EA cells.

These and previous observations reflect several varieties of naturally-occurring cytotoxins in human serum, whose function is the destruction of cells genetically alien to the host.

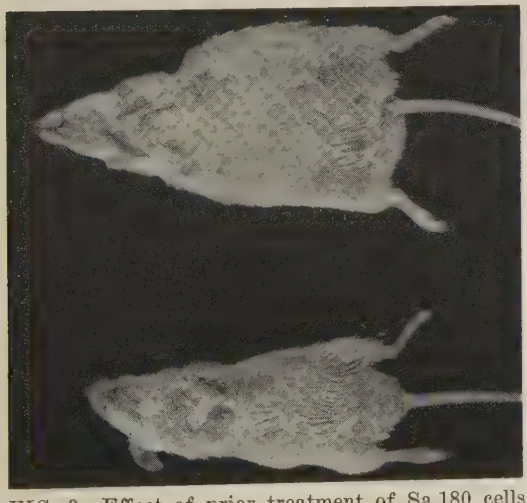


FIG. 3. Effect of prior treatment of Sa 180 cells with Serum and Serum △ on ability to produce ascites tumors. The large mouse had been inoculated 14 days previously with Sa 180 cells preincubated with Serum △. Autopsy revealed massive ascites tumor. The other mouse had been simultaneously inoculated with the same number of cells which had been incubated with normal human serum. Autopsy at 14 days revealed no evidence of ascites tumor.

TABLE II. Effect of Adsorption of Serum with Sa 180 Cells on Cytotoxicity for Sa 180 Cells and Components of C′.

No. adsorbing cells/ml serum	Cytotoxicity (%)*	C′ component titers (units/ml serum) C′1	C′2	C′3	C′4
0 (control)	100	1920	120	120	480
$.045 \times 10^7$	"	960	60	"	320
.090 "	"	"	"	"	"
.18 "	"	"	"	"	"
.37 "	98	"	"	"	"
.75 "	80	"	"	"	"
1.5 "	70	"	60	80	240
3.0 "	42	"	"	80	"
6.0 "	34	"	40	120	"
12.0 "	24	1920	"	80	160

* % of eosin-stained cells.

Summary. (1) Thermolabile, non-dialyzable cytotoxins are present in normal, human serum for Ehrlich ascites and Sarcoma 180 cells. (2) Similarities and differences of these cytotoxins and those active against atypical human cell strains U12 and HeLa are described. (3) Data are presented showing relationship of these cytotoxins to serum complement.

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Received November 16, 1959. P.S.E.B.M., 1960, v103.

Effect of Triethylenemelamine on Reproductive Capacity of Mouse Spermatozoa.* (25514)

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Triethylenemelamine (TEM) produces temporary sterility in male rats(1) as evidenced initially by early reduction in size of litters produced by females with which they are mated, and later by complete sterility. Smaller litters are accounted for by radiomimetic action of TEM in causing germinal epithelium of testes to produce so called(1) "sterile" spermatozoa which induce early embryonic death(1,2). Depending upon dose, TEM can affect germinal epithelium by causing histologically demonstrable damage to spermatogonia or by altering germinal cells in more subtle fashion so that spermatozoa are unable to produce viable offspring(2). Information on duration of spermatogenesis and passage of free spermatozoa(3,4) and available data on intervals between drug treatment and mating(1,2) support the view that the adverse effect on male fertility may be caused by injury to spermatozoa, as well as germinal epithelium. This communication presents evidence of this direct effect of TEM on reproductive capacity of mouse spermatozoa in reducing not only litter size but also per cent fertility, as tested by artificial insemination.

Materials and methods. Forty-four C57 male brown mice weighing 26 to 29 g were injected intraperitoneally each of 5 consecutive days with 0.006 mg TEM in 0.25 ml normal saline. This corresponds to the dose which reduced fertility and caused structural alterations in rat testes(2). Thirty-four were used in artificial insemination, 10 for measurements of density and additional observations on motility. Artificial insemination, after Kile(5), was used to evaluate reproductive capacity of spermatozoa from control and TEM treated males. Females of known fertility, kept in cyclic estrus by controlled lighting, were inseminated one to 3 hours following mating with vasectomized males. Both sexes were of BALB/c strain mice. After mid-ventral laparatomy, 0.1 ml of the spermatozoal suspension from a C57 male was introduced through the wall of each uterine horn with a 27 gauge hypodermic needle. The genetic label of eye color, carried by the spermatozoa of the C57 brown males, served as detectable insurance that all fetuses, both control and experimental, resulted from artificial insemination. Inseminated females were killed by etherization and autopsied on what was the nineteenth day of gestation in pregnant mice. TEM treated males were sacrificed by etherization 3 days after last injection. Testes were removed, fixed in Bouin's solution, and subsequently processed for histological observations on 5 μ paraffin sections stained by PAS - Weigert's hematoxylin technic. The cauda epididymides and the pair of ductus deferentes were removed from each animal. Each pair of structures was placed separately in depression slides containing 0.2 ml of a yolk-citrate-Locke's (YCL) medium(6). Spermatozoa were obtained from the epididymides by mincing with fine scissors, those from the ductus deferentes by stripping with blunt flat forceps. Following a 5 minute wait, each suspension of free spermatozoa was drawn up into a 0.25 ml syringe for insemination. The remainder of the sample was used in triplicate readings at 37°C on per cent motility. Type of motility and obvious abnormalities were noted. Density measurements were made with a blood counting chamber(7). Forty-nine untreated C57 brown mice served as controls.

From each of 8 mice, spermatozoa from the left cauda epididymis and ductus deferens were placed in YCL and those from the right side placed in YCL which contained the same concentration of TEM as given in a single injection to the other experimental mice. The

* This investigation was supported in part by Division of Research Grants, Nat. Inst. Health.

† Part of this work was completed while at American Found. for Biol. Research, Madison, Wisc.

TABLE I. Results of Artificial Insemination with Spermatozoa from Male Mice Treated with Triethylenemelamine.*

Spermatozoa	No. of females Insem-inated	Preg-nant	% fer-tility‡	No. of fetuses Total	Avg litter
Treated					
Ductus deferentes	34	3	9	5	1.7
Cauda epididymides	21	7	33	12	1.7
Total	55	10	18	17	1.7
Untreated†					
Ductus deferentes	30	20	67	100	5.0
Cauda epididymides	28	15	53	72	4.8
Total	58	35	60	172	4.9

* Inj. intraper. with 0.2 mg/kg TEM for each of 5 consecutive days.
† Control group.
‡ Nearest whole number.

type and per cent motility of spermatozoa from both sides of the same animals were then compared.

Results. Artificial insemination with spermatozoa from the cauda epididymides and ductus deferentes of mice injected each of 5 consecutive days with a dose of TEM equivalent to 0.2 mg/kg body weight, resulted in a marked decrease in per cent fertility, as well as reduced litter size. Inseminations with spermatozoa from the ductus deferens resulted in a greater lowering of per cent fertility than those from the epididymis (Table I). Per cent motility and density of spermatozoa from animals so treated with TEM were within the range of values found in control mice (Table II). No evidence of induced structural abnormalities in spermatozoa was observed. TEM treatment caused an average loss of one gram of body weight.

Histological comparisons of testes from untreated and TEM treated males revealed no TEM induced structural deviations from normal spermatogenesis.

The type, speed, and per cent motility of spermatozoa treated *in vitro* with the same concentration of TEM as used in one injection, appeared unchanged from the normal in the comparisons between treated and untreated cells from the same animal.

Discussion. We agree that an adverse effect of TEM on spermatozoa is reflected in reduced litter size of inseminated females(8). In addition, we find that per cent fertility also is diminished although number, motility, and appearance of spermatozoa remain unchanged, in the absence of testicular structural abnormalities.

Eight days elapsed between first injection of TEM and insemination, while 7 to 8 days are required for spermatozoa to traverse the epididymis in passing from testis to ductus deferens(4). Thus, inseminated samples from the ductus deferentes probably contained spermatozoa which were in the ductus deferentes, in the epididymides and in the testes, at the time TEM was first injected. Epididymal samples inseminated probably included spermatozoa which were late spermatids at this time. Therefore, results (Table I) do not support the view(8) that spermatids are more sensitive to TEM than are spermatozoa. This may be explained if the stage at which spermatids are treated is critical. Early and not late spermatids may be more sensitive than spermatozoa to TEM.

A double action of TEM in reducing male fertility is suggested. Reduction in total number of pregnancies as well as size of litters produced (Table I) makes possible both prevention of fertilization and early embryonic death. There was no evidence of increased implantation sites or reabsorptions in treated mice.

Spermatogenesis in mice is apparently more

TABLE II. Per Cent Motility and Density of Spermatozoa from Male Mice Treated with Triethylenemelamine.*

	Spermatozoa	Treated Avg	Treated Range	Untreated Avg	Untreated Range
% motility†	Ductus deferentes	58	20-70	56	25-70
	Cauda epididymides	51	20-70	52	20-70
Density‡	Ductus deferentes	11.2	6-16	10.9	7-14
	Cauda epididymides	13.3	7-23	13.1	10-17

* Inj. intraper. with 0.2 mg/kg for each of 5 consecutive days.
† Data from 44 treated and 49 untreated male mice.
‡ Data from 10 treated and 10 untreated male mice in millions of cells/cc.

sensitive to x-rays than in rats, as reflected by induced oligospermia with the same low dose (9,10). Since TEM is a radiomimetic drug (1,2), it is surprising that, unlike the rat(2), mice treated with the same dose did not show structural defects in spermatogenesis. However, our interval betwen TEM treatment and fixation of testes may not have been long enough for manifestation of changes.

Summary. Triethylenemelamine (TEM) has been shown to affect adversely the reproductive capacity of male mice by acting on and through spermatozoa without altering their number, motility, or appearance. Females artificially inseminated with spermatozoa from the ductus deferentes and cauda epididymides of treated males showed a marked reduction in per cent fertility as well as litter size. Spermatozoa appeared more sensitive to TEM than late spermatids. No obvious structural changes were induced in testicular elements during treatment. The motility of spermatozoa was unchanged by exposure to a high concentration of TEM *in vitro*.

The authors acknowledge Dr. T. P. Lin for advice on AI technic and Marjorie Crandall for capable assistance.

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Received November 16, 1959. P.S.E.B.M., 1960, v103.

Pyridoxal Phosphate in Plasma and Leukocytes of Normal and Pregnant Subjects Following B_6 Load Tests.* (25515)

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The concentration of pyridoxal phosphate, the coenzymatically available active form of Vit. B_6, has been estimated in leukocytes of normal subjects(1,2), and in those of pregnant women and cord blood(2), using a modification of the manometric method of Umbreit *et al.*(3). Boxer(1) also tried to utilize the method for determination of pyridoxal phosphate in total blood, but did not find measurable quantities in most human bloods unless the subjects were given oral doses of Vit. B_6. Comparisons of plasma and leukocyte values are herein described. Normal subjects, pregnant women and cord blood of newborn babies were studied. In addition, load tests were performed in normal controls, in a group in last trimester of pregnancy and in women who delivered their babies one day prior to test. We investigated whether load tests would give information not readily obtainable by single plasma or leukocyte determinations.

Method. Whole blood contains interfering substances necessitating use of diluted blood (1). Preliminary experiments were, therefore, undertaken to establish optimal dilution for performance of test. Plasma diluted 1:6, 82% of added Vit. B_6 could be recovered, whether or not its initial content was normal or relatively large, *e.g.,* obtained from individuals who had ingested a large amount of Vit. B_6. In contrast, in whole blood diluted 1:6, only 68%, and in packed red cells, only 61% of added pyridoxal phosphate could be recovered (Table I). For simultaneous estimation of the vitamin in leukocytes and plasma, 5 ml

* This work was aided by grant from Nat. Vitamin Fn.

TABLE I. % Recovery of Added Pyridoxal Phosphate Standard (4 mγ). (Each figure is average of at least 5 determinations.)

Analysate	Undiluted	1:3	1:6
Plasma	13.7	61	82
Whole blood	7.2	59	68
Red cells	0	50	61

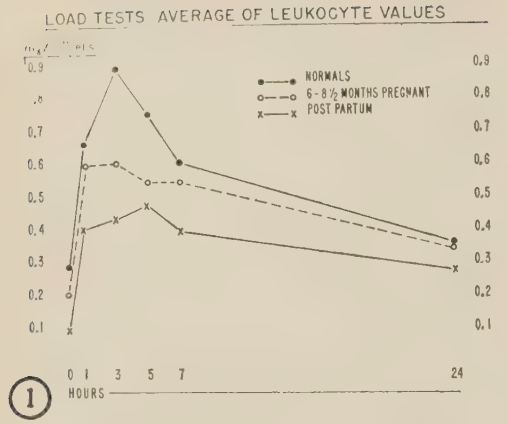
of venous blood was collected in a tube containing about 0.02 g of sodium versenate. Leukocytes were isolated using freeze-dried phytohemagglutinin prepared from navy beans. Separated leukocytes were treated as previously described. The plasma obtained was diluted 1:6 with distilled water. The assay method was based on coenzyme function of pyridoxal phosphate in a tyrosine decarboxylase system. Tyrosine decarboxylase prepared from Vit. B_6-free preparation of *Streptococcus fecalis,* was obtained from Dept. of Bacteriology, Rutgers Univ., New Brunswick. Minor changes were made in technic as described by Boxer *et al.*(1). Larger flasks (volume 15 ml) were used so that larger samples could be utilized. One cc of the diluted sample was put into flasks together with 1 cc of acetate buffer pH 5.5, ½ cc of L-tyrosine suspension of 25 g/100 ml buffer and ½ cc of a suspension of 4 mg tyrosine decarboxylase/ml water. This amount was considered optimal since increase in concentration of the decarboxylase suspension did not result in increase in amount of CO_2 given off. Instead of n-heptane, Brodie's solution was used as manometer fluid. All determinations were done in duplicate within 12 hours after sample had been obtained. Even at 4°C, some loss of Vit. B_6 occurred after 24 hours in plasma and after 48 hours in leukocytes. When duplicate manometer movements were used for calculation of standard deviation of all plasma results, this value came to ± 0.5 mγ/ml.

Results. 1. *Normal values*: concentration of B_6 in plasma and leukocytes was determined in 27 normal men and 20 normal women. The samples were usually obtained in the morning. No vitamin preparations had been taken by these individuals for at least 3 months prior to the test. In men, range of pyridoxal phosphate in leukocytes was 0.15 to 0.36 mγ/million cells with average of 0.23 (± 0.05). In women, the range was 0.14 to 0.30 mγ average 0.22 mγ (± 0.05/million cells. Plasma values were 5.2 to 16.2 mγ/ml, average 10.5 (± 2.5) in men and 5.2 to 12.0 mγ/ml, average 8.4 (± 2.5) in women. In these figures are not included 2 plasma determinations of 33 mγ/ml in a man and 18 mγ/ml in a woman. No obvious reason for these exceedingly high values could be found.

In 4 men and 2 women B_6 levels were measured 2-4 times at weekly intervals. Considerable variations in apparently healthy individuals were observed (Table II). Leukocytes and plasma levels showed, within a relatively short time, variations extending through the whole range in normal individuals.

2. *Mothers at term and cord blood of their babies*: Venous blood was obtained from 19 mothers at time of delivery, and from cord blood of their babies. Leukocyte values in maternal blood varied from 0.02 to 0.19 mγ, average 0.09 mγ, and in cord blood from 0.11 to 1.22 mγ, average 0.47 mγ. Respective plasma values varied from less than 2 to 8.6 mγ/ml, average 4.3 mγ/ml, and in cord blood from 10.8 to 49.2 mγ/ml, average 23.2 mγ. The difference between cord blood and blood of mothers is highly significant ($p<0.01$). Also significantly lower leukocytic and plasma values were noted in women at time of delivery ($p=0.075$) compared to normal women.

3. *Load Tests*: Three groups of individuals were used consisting of 9 normal controls, 5 men and 4 women, 7 women in third trimester of pregnancy, and 9 women 24 hours after delivery. Following withdrawal of a venous blood specimen in the morning, the experimental subjects were given an oral dose of 100 mg of pyridoxine hydrochloride (Merck). Blood samples were withdrawn after 1, 3, 5, and 7 hours, and final sample 24 hours after administration of the vitamin (Fig. 1, 2). In all 3 groups, peak values were reached on the average somewhat earlier in leukocytes than in plasma. The greatest rise in both leukocytes and plasma occurred in normal controls, and the lowest in women at term. Women in last trimester of pregnancy showed intermediate values.



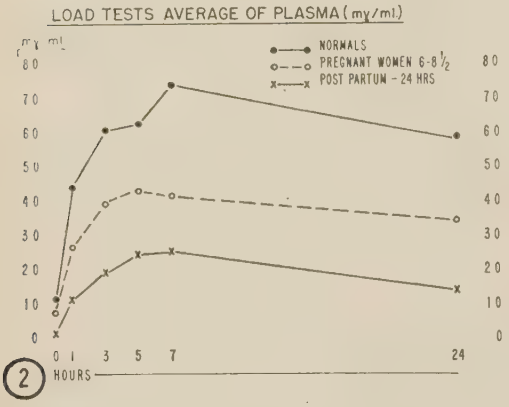


FIG. 1. Avg of pyridoxal phosphate values in leukocytes before and after oral administration of 100 mg pyridoxine hydrochloride in a group of normal controls, women 6 to 8½ mo pregnant and 24 hr post partum.

FIG. 2. Avg of pyridoxal phosphate values in plasma before and after oral administration of 100 mg pyridoxine hydrochloride in a group of normal controls, women 6 to 8½ mo pregnant and 24 hr post partum.

Twenty-four hours after administration of the vitamin, level of leukocytic B_6 in all 3 groups was slightly higher than normal average values. There was no statistically significant difference in amount of Vit. B_6 present in leukocytes of controls and the 2 groups of pregnant women. In contrast, plasma values after 24 hours were very high in control subjects averaging 60 mγ/ml while in women at term they averaged only 15 mγ/ml. In women in third trimester of pregnancy, 24 hour plasma averaged 35.5 mγ/ml. The difference of these values between pregnant women and normal controls, ($p = 0.05$) and between women after delivery and normal controls ($p < 0.01$) is statistically significant. It is interesting to note that preload determinations in women in third trimester of pregnancy revealed normal values for both plasma and leukocytes in 6 out of 7 cases.

Comment. Although average plasma values were somewhat lower in normal women than men, this difference is of questionable significance. Repeated weekly determinations of pyridoxal phosphate in plasma and leukocytes in the same individuals revealed considerable variations in both estimations (Table II). A close parallelism of leukocyte and plasma values was, on the other hand, consistently observed in blood of women in labor and in cord blood of their babies. Both plasma and leukocytes of the cord contained on the average 5 times as much Vit. B_6 as did maternal blood.

Vitamin load tests revealed considerably lower peak values in both plasma and leukocytes, in women at term and to a lesser degree in later pregnancy in comparison to normal controls (Fig. 1, 2). Twenty-four hours after administration of test dose of Vit. B_6, plasma values remained high in normal controls, but were very low in delivering women, indicating that pyridoxine is taken up avidly by the maternal tissues. That the amount of leukocytic vitamin was not significantly different in the 3 groups 24 hours after pyridoxine had

TABLE II. Result of Weekly Determinations of Plasma and Leucocyte Pyridoxal Phosphate Levels in 6 Control Subjects.

		Plasma, mγ/ml					Leukocytes, mγ/million cells				
Subject	Sex	1	2	3	4	Range	1	2	3	4	Range
E.L.	♂	12.8	10.7	10.0	9.4	9.4-12.8	.15	.26	.27	.18	.15-.27
H.M.	♂	15.2	11.4			11.4-15.2	.21	.23			.21-.23
T.V.	♂	11.0	5.2	7.2	8.1	5.2-11.0	.34	.16	.22	.15	.15-.34
A.N.	♂	10.5	8.8	10.7	5.3	5.3-10.7	.17	.33	.18	.24	.17-.33
C.F.	♀	6.5	6.7	5.9		5.9- 6.7	.23	.21	.14		.14-.23
P.T.	♀	7.4	7.6	5.3		5.3- 7.6	.23	.19	.16		.16-.23

been administered suggests that normal tissue level of vitamin had been restored for the time being, even in the most deficient group.

At termination of normal pregnancy, B_6 values are significantly depressed in maternal leukocytes and plasma. In blood of women in last trimester of pregnancy, however, normal average amounts of the vitamin are found although these individuals have a distinct abnormality in tryptophane metabolism which can be readily corrected by administration of pyridoxine(4). This relative pyridoxine deficiency can be demonstrated by the Vit. B_6 load test as described here. From a practical point of view, determination of plasma values 24 hours following administration of Vit. B_6 may be useful in evaluating the B_6 saturation of tissues in clinical subjects.

Summary. A method for determination of pyridoxal phosphate in human plasma is described. The amount of coenzymatically active form of Vit. B_6 was determined simultaneously in blood of normal subjects, delivering women, and in cord blood of their babies. In general, amounts of pyridoxal phosphate in plasma and leukocytes showed parallel changes. They were low in maternal blood, and high in cord blood. Following oral administration of 100 mg pyridoxine hydrochloride, increases were observed in both plasma and leukocytic B_6 values within a few hours. Considerably higher peaks were seen in normal controls as compared to women in last trimester of pregnancy and at time of delivery. The most significant difference between pregnant and nonpregnant subjects was noted in plasma values 24 hours following administration of vitamin. The possible practical application of these findings for evaluation of a latent B_6 deficiency in clinical subjects is suggested.

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Received November 18, 1959. P.S.E.B.M., 1960, v103.

Effects of Epinephrine on Toxicities of Several Local Anesthetic Agents. (25516)

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Apparently the opinion is held generally that inclusion of a vasoconstrictor, such as epinephrine, in solution with local anesthetic, such as procaine, affords considerable protection against danger of systemic intoxication by the anesthetic when such solution is injected into soft tissues. This protective action is said to result from slower, more gradual absorption of the anesthetic because of localized vasoconstriction, so that mechanisms for detoxication and elimination of the anesthetic are more nearly able to keep pace with absorption. This, then, prevents development of excessively high blood stream concentration of the toxic agent. Goodman and Gilman(1) state that this is true, although no reference is made to experimental verification. Campbell and Adriani(2) state that systemic toxicity of procaine after infiltration is reduced 30% or more by addition of 1:100,000 epinephrine to the solution, although, no source is given for the information. Sollmann(3) holds a similar view supported by reference to observations by Hatcher and Eggleston(4), Eggleston and Hatcher(5), Zaus and Moody(6), and Beutner, Prusmack, and Miller(7). The conclusions of Hatcher and Eggleston and by Eggleston and Hatcher were based on too few observations, without adequate controls. Since only 2 animals were used, both surviv-

ing, their experimental results hardly justify such conclusion. Zaus and Moody state that their data "rather definitely prove that epinephrine, as used in local anesthetic solutions, is a favorable agent" for it affords protection against procaine. However, their experiments were with direct intravenous infusion of these agents, not with injection into soft tissues. Here, again, the conclusion applied to injection into soft tissues as well, is not justified by published results. Beutner *et al.* found that epinephrine in concentration of 1:50,000 minimized or prevented convulsions in rabbits following intramuscular injection of procaine in doses which, otherwise, caused severe convulsions. But determinations of fatal doses with and without epinephrine were not reported. In contrast Hatcher and Eggleston report that Heineke and Laewen in 1905 observed no appreciable difference in toxicity of procaine on subcutaneous injection into rabbits, with or without epinephrine. Tainter and Throndson(8) found that neither epinephrine (1:10,000 and 1:50,000) nor Neosynephrine (1:2500) caused a decrease in toxicity of procaine on intraperitoneal injection into rats, but a slight though statistically unimportant increase. Cobefrin (1:10,000) caused definite increase in toxicity. Gurd and Sachs (9) very carefully studied the effect of epinephrine on toxicities of cocaine and procaine after subcutaneous injection in mice. They found that relatively low concentrations of epinephrine (1:100,000 or less) had no significant influence on toxicity of either agent, but higher concentrations (1:85,000 for cocaine; 1:50,000 for procaine) significantly increased toxicities of both agents. Investigations by various authors in support of protection contained so little of a positive nature, while investigations which bear directly on the subject received little attention. There is good evidence that appreciable protection is obtained following *intravenous* injection, but this is a different matter. Also, there appears to be no reason to question the widespread belief that inclusion of a vasoconstrictor makes possible satisfactory anesthesia of greater duration with less anesthetic agent than would be possible without the vasoconstrictor. But, this too is a different matter. In view of the confusion which exists with respect to protection following infiltration of local anesthetics into soft tissues, further investigations were considered desirable.

Material and methods. It has long been recognized that local anesthetics undergo changes within the animal, these changes being referred to as "detoxication." Brodie, Lief, and Poet(10) demonstrated that in the human some extra-hepatic mechanism, probably associated with blood, is responsible for the greater part of detoxication of procaine. Kalow(11) identified this mechanism, so-called "procaine esterase," as plasma or serum cholinesterase (pseudocholinesterase). It has been recognized also that rate of detoxication of even a single agent varies greatly from one species to another. Thus, Aven, Light and Foldes(12) found that mouse serum is much more active in hydrolyzing procaine than that of most other commonly available laboratory animals, and that it approaches, but does not equal, human serum. Unfortunately, procaine appears to be the only agent investigated with respect to rates of hydrolysis in sera other than the human. However, on the basis of present evidence, it appears that the mouse is the preferred species for many investigations dealing with toxicity of local anesthetics. The ready availability of large numbers of uniform stock is an additional advantage. Accordingly, male white mice of 20-30 g body weight were used. Five representative anesthetic agents were selected. Three of these, procaine, tetracaine, and piperoceaine are esters, and are subject to hydrolysis by human plasma esterases though at different rates (Kalow). The other agents, lidocaine and dibucaine, are amides, and are not hydrolyzed at measurable rates by human plasma esterases. If vasoconstriction at site of injection slows absorption of the anesthetic agent, then one would anticipate greater protection by a vasoconstrictor against agents which are hydrolyzed rapidly, and less protection against those which are hydrolyzed slowly or not at all. With exception of lidocaine, all anesthetic solutions were prepared from hydrochloride crystals. A crystalline preparation of lidocaine hydrochloride was not readily available, so commercial 2% solution was used. Con-

TABLE I. Effects of Epinephrine on Toxicities of Several Local Anesthetic Agents.

Agent	Epinephrine conc. × 1000	LD_{25}	$LD_{50} \pm$ S.E.	LD_{75}	Slope $(\log mg)^{-1}$
Procaine	0	616*	695 ± 17	785	12.8
	1:500	316	602 ±122	1149	2.40
	1:100	314	539 ±104	924	2.88
	1:50	399	453 ± 26	765	4.77
Piperocaine	0	371	615 ± 84	1021	3.07
	1:500	481	706 ± 54	1035	4.06
	1:100	320	441 ± 39	608	4.83
	1:50	365	447 ± 27	546	7.80
Tetracaine	0	34.6	40.6 ± 2.1	47.7	9.6
	1:500	40.8	51.0 ± 4.0	58.7	8.5
	1:100	56.8	64.8 ± 2.8	74.1	11.6
	1:50	69.0	76.6 ± 4.0	89.8	9.8
Lidocaine	0	113	163 ± 16	235	4.24
	1:500	163	185 ± 6.3	210	12.2
	1:100	178	195 ± 6.2	214	16.8
	1:50	153	198 ± 14	256	6.04
Dibucaine	0	24.6	25.4 ± .28	26.2	48.9
	1:500	23.4	24.7 ± .44	26.1	27.9
	1:100	25.5	26.6 ± .36	27.8	36.2
	1:50	24.8	28.1 ± .96	31.7	12.5

* All doses expressed as mg/kg of hydrochloride.

centrations were such that a constant volume of 0.01 ml/g body weight was injected. All injections were made beneath the skin of lumbar region of the back. Epinephrine was added to solutions to produce concentrations as indicated in Table I. Dose-response curves were determined by at least 3 points, usually 4 or 5. As a rule, 15 to 25 animals were used in each group; but in a few only 10. LD_{50}'s and standard errors were calculated by minimum normit chi-square(13).

Results. The results shown in Table I are at variance with the view that inclusion of a vasoconstrictor reduces systemic toxicity. With only one agent, tetracaine, of the 5 studied, was this found to be true. Early in the investigation it became apparent, especially with procaine, that inclusion of epinephrine may lead to a marked change in slope of the dose-response curve. Not only the LD_{50}'s for various agents and their mixtures with epinephrine, but also the slopes and doses for responses at both 25% and 75% mortality levels are therefore included in the Table. With procaine there appears to be no significant reduction in toxicity at any response level or with any concentration of epinephrine with the possible exception of the LD_{75} obtained with 1:500,000 epinephrine. On the contrary, there is a strong suggestion of increased toxicity with increase in concentration of epinephrine, statistically significant at the LD_{50} level with 1:50,000 epinephrine. With piperocaine, also, there is no evidence of protection but rather the suggestion of an increase in toxicity under most conditions. With both lidocaine and dibucaine there is evidence of a slight reduction in toxicity under certain conditions, but this is of doubtful importance.

Summary. With only one agent (tetracaine) of the 5 studied, was addition of epinephrine accompanied by a highly significant reduction in systemic toxicity. With the 4 remaining agents toxicity was not altered appreciably under most conditions, although procaine containing 1:50,000 epinephrine seems more toxic than procaine alone.

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Received November 19, 1959. P.S.E.B.M., 1960, v103.

Adrenal Sensitivity to ACTH as a Function of Time after Hypothalamic Lesion and after Hypophysectomy.* (25517)

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The usually available criteria for endogenous discharge of ACTH are indirect and are based on corticotropic stimulation of the adrenal cortex. When studying hypophysiotropic activity of preparations of corticotropin releasing factor (CRF) in animals with "effective" hypothalamic lesions, one should therefore know the sensitivity of the adrenals to ACTH. Considered "blocked" in their ACTH discharge when they show no evidence of adrenocortical stimulation upon exposure to stress, these animals might simply be less sensitive to ACTH than normal. Adrenocortical sensitivity to ACTH has been studied at various time intervals ranging from 24 hr to 60 days after stereotaxic placement of a lesion in the median eminence and demonstration of its effectiveness to inhibit stress-induced ACTH release. For comparison, adrenal sensitivity to ACTH was also studied in hypophysectomized animals.

Materials and methods. A. *After hypothalamic lesion.* Two hundred sixty animals (rats, male, body weight 230-250 g, Holtzman Farms, Houston) were used. A lesion of the bulbar part of the median eminence was made (Fig. 1), using a high frequency generator, the electrodes being placed with a modified Krieg-Johnson stereotaxic instrument. Brains of *all* animals were preserved at autopsy and a description of lesions from frozen sections is available for each animal. Eighteen to 19 hours after placement of the lesions, all animals were tested for block of stress-induced ACTH discharge as previously described(1). Only animals with "effective" lesions (60 out of 260) were saved for experiments reported here. Twenty-four hours after lesioning, 3 groups of animals were administered 0.5, 1.0, 1.5 mU ACTH USP Reference Standard (I.V., in 0.2 ml of 0.01 N HCl in saline). Adrenocortical response to 1 mU ACTH USP Reference Standard administered as above was investigated at 2, 4, 7, 9, 20, 30 and 60 days following placement of hypothalamic lesion. Persistence of inhibition of stress-induced ACTH-release(3) was reevaluated 6 hours before injection of ACTH in all animals used after fourth post-lesion day. In all cases adrenocortical stimulation produced by ACTH was assessed by measurement of peripheral plasma free corticosteroids 15 minutes after injection of the solution of adrenocorticotropin(2,3). The animals were then sacrificed and weight of adrenal glands recorded.

B. *After hypophysectomy.* One hundred fifty-six animals (rats, male, body weight 175-200 g, Holtzman Farms) were hypophysectomized. Three groups were administered 0.25, 0.5, 1.0 mU ACTH USP Reference Standard (as above), 24 hours later. Animals of other groups were similarly injected with 1 mU

* This work supported by U.S.P.H.S. grants and U.S.A.F. contract.

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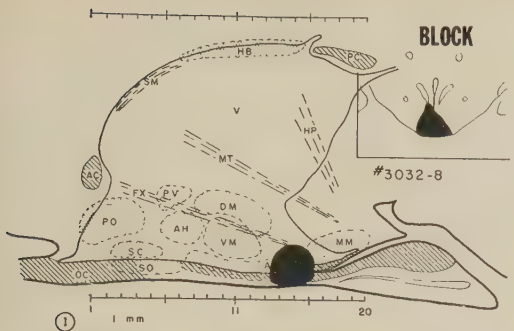

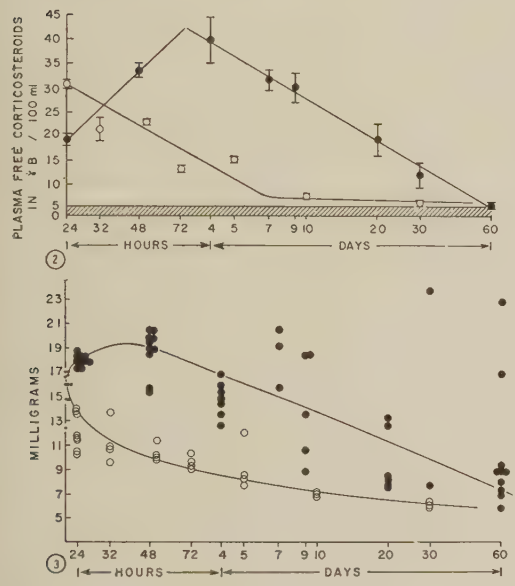

FIG. 1. Typical hypothalamic lesion (see text).

FIG. 2. Adrenocortical response to 1 mU ACTH at various time intervals after hypothalamic lesion (●) or hypophysectomy (○). Vertical bars through each circle represent 1 stand. error. (No. of animals/group can be calculated from Fig. 3.) Stippled area represents background reading in fluorometric method for plasma corticosteroids(2).

FIG. 3. Adrenal weights (left gland) after hypothalamic lesion (●) or hypophysectomy (○). (•), absolute controls of same experimental series.

ACTH, 32 and 52 hours, 3, 5, 10 and 30 days after hypophysectomy. Adrenocortical stimulation following ACTH was assessed as above (2,3), and adrenal weights recorded.

Results. Table I shows response of animals to multiple doses of ACTH, 24 hours after hypophysectomy and 24 hours after placement of an effective lesion. The animal with the hypothalamic lesion has an adrenal sensitivity to ACTH which is slightly lower than that of the hypophysectomized. Fig. 2 shows adrenal response of hypophysectomized and lesioned animals when injected with 1 mU ACTH USP Standard at various intervals after hypophysectomy or hypothalamic lesion. In the rat with hypothalamic lesion there is an increased response to ACTH for a few days (as compared to that observed at 24 hours post lesion). Subsequently, the response to ACTH decreases exponentially to completely disappear at 60 days after the lesion. After hypophysectomy, the adrenal response to 1 mU ACTH decreases rapidly to become asymptotically nil after 10 days. However 30 days post-hypophysectomy, there is still indication of a minute response to 1 mU of ACTH as judged by measurement of the plasma corticosteroid levels; this was confirmed by measurement of concentration of corticosterone in adrenals of the same animals (not reported here). Fig. 3 shows weights of adrenals as a function of time after hypophysectomy or hypothalamic lesion. Atrophy of the adrenal gland is clearly observable as early as 24 hours after hypophysectomy. It progresses linearly as a function of time. The adrenal of animal with effective hypothalamic lesion increases in weight in the first 48 hours; it then decreases in weight as a linear function of time. There are however considerable variations around mean adrenal weight calculated for each group, even though the responsivity to ACTH of these adrenals is quite constant within each group as exemplified by the small standard error of mean responses (Fig. 2).

Discussion. The progressive decrease of adrenal sensitivity to ACTH in animals with effective hypothalamic lesions, makes it desirable to use only an acute preparation as a

TABLE I. Adrenocortical Response to Multiple Doses of ACTH 24 Hr after Hypothalamic Lesion (A) or Hypophysectomy (B).

	No. animals	Plasma B, γ/100 ml, after stress	mU ACTH	Plasma B, γ/100 ml, after ACTH
A	4	12.6 ± 1.3*	.5	12.7 ± 1.7
	10	6.7 ± 1.8	1.0	19.4 ± 2.3
	5	13.6 ± 2.8	1.5	27.1 ± 2.7
B	6		.25	19.1 ± 1.7
	6		.5	24.0 ± .5
	6		1.0	30.9 ± 1.9

* Stand. error of mean.

test for CRF. In view of the increased response to ACTH, 2-4 days post-lesion, this period would seem to be an even better time schedule (than 1 day after lesion) for CRF assays. Since, 24 hours post-lesion, the animal has a sensitivity to CRF similar to that of morphine-nembutal blocked animal(see 1) which in turn has an adrenocortical sensitivity to ACTH similar to that of the 24-hour hypophysectomized rat, it is probable that 24 hours after hypothalamic lesion a greater sensitivity of the pituitary to CRF compensates for a submaximal adrenal response to ACTH. The increased adrenocortical response to the standard 1 mU dose of ACTH, as observed in early days after hypothalamic lesion, may be related to increased adrenal weight. This transient increased adrenal weight is likely related to trauma involved in surgical procedure at lesioning; similar adrenal weights were noted in animals of the same experimental series submitted to sham hypothalamic lesion.

It is interesting that up to 30 days after hypothalamic lesion, circulating free corticosteroids are still detectable in peripheral plasma (4). These levels, however, should not be necessarily considered as true correlates of concentration of any circulating ACTH; they may simply reflect impaired conjugation mechanisms. Taken in conjunction with maintenance of adrenocortical response to ACTH, still significant 30 days after the hypothalamic lesion and considerably higher than that observed at the same time following hypophysectomy, these data would indicate some paralytic secretion of adenohypophysial ACTH after placement of the hypothalamic lesion. This residual secretion appears to diminish exponentially with time (Fig. 2) and to have practically disappeared by the 60th day post lesion (no adrenocortical response to exogenous ACTH). In animals with this type of hypothalamic lesion, the corresponding hypocorticoidism is therefore unable to elicit the well known reciprocal hyper-secretion of ACTH.

Through latest time interval studied here, the adrenal gland of the hypothalamic animal remains statistically heavier than that of the hypophysectomized; variations around mean adrenal weight of each group are however considerable (Fig. 3). Since large adrenals as late as 60 days post hypothalamic lesion, are unable to respond to exogenous ACTH by increased corticoidogenesis, it is difficult to visualize weight maintenance solely on the basis of circulating ACTH. Two likely substances of hypophysial origin which might explain this adrenotrophic effect after hypothalamic deafferentiation of the pituitary are prolactin and growth hormone. This hypothesis will be discussed in another report.

Summary. The adrenocortical response to injected ACTH USP Standard has been studied as a function of time in rats after hypophysectomy or placement of a lesion in median eminence of the hypothalamus. In either case, there is an exponential decrease in sensitivity to ACTH of the adrenal gland, as a function of time. This can be observed in the presence of large adrenal glands, in animals bearing a hypothalamic lesion. The progressive decrease of adrenal sensitivity to ACTH in the lesioned animal makes it desirable to use only an acute preparation as a test for CRF.

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Received November 20, 1959. P.S.E.B.M., 1960, v103.

Virulence of a Nutritional Mutant of *Vibrio comma.* (25518)

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It was first shown by Bacon *et al.*(1) that purine requiring mutants of *Salmonella typhosa* are less virulent for mice than their prototrophic parents when injected intraperitoneally. The mutants which they employed were obtained by the technic of Lederberg and Zinder(2) and Davis(3). Using similar procedures, Garber *et al.*(4) reported that a similar situation existed in strains of *Klebsiella pneumoniae.* Subsequently Formal *et al.*(5) found that a naturally occurring mouse-avirulent typhoid mutant which on one occasion had been isolated from the Panama carrier also required purines for growth. The fact that purines are either absent or present in limiting concentration in peritoneal cavity of the mouse offers a logical explanation for the relative avirulence of purine requiring strains when administered intraperitoneally to mice. For some time it has been known that laboratory strains of *Vibrio comma* differ in their capacity to infect mice fatally(6). Our purpose was to determine whether this difference might be attributed, at least in part, to the fact that an avirulent culture has a purine requirement for growth while a more virulent strain does not. In addition, we considered it pertinent to compare ability of strains to infect mice fatally following challenge by intraperitoneal route and guinea pigs after oral administration.

Materials and methods. Two strains of *V. comma,* obtained from the type culture collection of Walter Reed Army Inst. of Research, were employed in our work; both were Inaba types. Strain 20-A-67 requires only glucose and inorganic salts for growth while strain 20-A-47 requires purines in addition to this minimal medium. A mutant of strain 20-A-47 which proved purine independent, was obtained by plating 10^9 cells of this strain on minimal medium and selecting a colony which grew out. This purine-independent variant has been designated 20-A-47 PI. Virulence tests in mice were performed by injecting 0.5 ml amounts of known numbers of organisms suspended in 5% hog gastric mucin into the peritoneal cavity of Bagg strain mice weighing 12 to 16 g. Equal numbers of males and females were used to test each dilution. When the effect of purines in virulence tests was examined, 10 mg xanthine and 3 mg adenine contained in 0.5 ml saline was injected intraperitoneally immediately after the challenge suspension. Animals not receiving purines received an equal volume of saline. Hartley strain guinea pigs were rendered susceptible to a fatal enteric infection following oral challenge by depriving animals of food for 4 days and feeding 125 mg calcium carbonate prior to and injecting 1 ml tincture of opium intraperitoneally following orally administered challenge(7,8). Mucinase tests were carried out using a procedure previously described (9).

Results. Results of virulence tests employing intraperitoneal challenges in mice are summarized in Table I. The LD_{50} of strain 20-A-47 which requires purines for growth is approximately 700 x 10^5 cells; when purines are inoculated into the peritoneal cavity at the same time as the challenge suspension, the LD_{50} is reduced to 4.1 x 10^5. The purine-independent mutant of strain 20-A-47 has an LD_{50} of 2.5 x 10^5 and as can be seen, the simultaneous inoculation of purines does not significantly increase its ability to cause a fatal infection. The LD_{50} of strain 20-A-67, which does not require purines to multiply, is 10x10^5 cells. The difference in the LD_{50} of strain 20-A-67 and the purine-independent mutant of strain 20-A-47 may be due in part to experimental error and perhaps to the fact that the wild-type culture of 20-A-67 employed, might have contained a fair proportion of cells requiring purines or similar substances for growth, thus decreasing the overall mouse virulence of the strain. On the other hand, the purine-independent mutant of strain 20-A-47 had been isolated from a mini-

TABLE I. Effect of Inoculation of Purine and Reversion to Purine Independence on Mouse Virulence of *Vibrio comma* Strain 20-A-47.

Challenge	Requires purines	No. of organisms inj.* 5×10^8	5×10^7	5×10^6	5×10^5	5×10^4	5×10^3	LD_{50}	S.E.
20-A-47	Yes	33/40	18/40	1/40	0/40	0/40		700 $\times 10^5$	270 $\times 10^5$
20-A-47 + purines†	"		37/40	36/40	22/40	8/40		4.1 "	1.5 "
20-A-47 PI	No		32/40	26/40	23/40	11/40	4/40	2.5 "	.9 "
20-A-47 PI + purine†	"		39/40	33/40	30/40	20/40	6/40	1 "	.4 "
20-A-67 (purine independent)	"		30/40	27/40	20/40	9/40	5/40	10 "	45 $\times 10^4$

* Organisms suspended in 5% hog gastric mucin.
† 10 mg xanthine and 3 mg adenine administered intraper. at time of challenge.

mal glucose plate and its population was perhaps more homogenous. We have had occasion over a period of a year to perform experiments similar to those summarized here and have noted that the actual LD_{50} values could vary considerably—perhaps as much as a thousand fold—but the values relative to each other were always approximately the same.

Table II gives a summary of results of experiments comparing ability of these strains to infect Hartley strain guinea pigs by the oral route. A single dose level of approximately $3x10^7$ cells was employed, since it was known from experience that a dose of this magnitude would fatally infect a large percentage of the animals when strain 20-A-67 is used as a challenge. The purine-dependent, mouse-avirulent 20-A-47 strain caused a 33.9% mortality while the purine-independent, mouse-virulent 20-A-47 PI strain produced a 30% mortality. On the other hand, 68.6% of the animals challenged with the wild-type purine-independent mouse-virulent strain 20-A-67 succumbed.

We have not been able to detect any other significant biological differences among strains 20-A-67, 20-A-47, and the purine-independent mutant of strain 20-A-47. They are similar antigenically and all have the capacity to produce mucinase in brain-heart infusion broth shake cultures; whether they have the ability to produce the enzyme *in vivo* is not known. When tested for their ability to produce mucinolytic enzymes in nutrient broth shake culture, however, mucinase was only detectable in the 20-A-67 supernatant. While this observation may be significant, additional work is necessary to establish its importance.

Discussion. The data presented demonstrate that the nutritional requirements of a culture of *Vibrio comma* may influence its ability to cause a fatal infection in mice following intraperitoneal inoculation. Thus, in this respect the requirement for purines by cholera strains affects their virulence for mice just as it influences the mouse virulence of strains of *Salmonella typhosa* and *Klebsiella pneumoniae*.

The results also indicate that the capacity of cholera cultures to infect mice fatally by the intraperitoneal route and guinea pigs by the oral route need not necessarily go hand in hand. It is likely that the organism must possess attributes additional to those needed

TABLE II. Deaths in Starved Hartley Strain Guinea Pigs Following Oral Administration of a Purine-Requiring Strain of *Vibrio comma* and a Purine-Independent Mutant Derived from It.*

Strain	Requires purines	Virulent for mice†	Deaths‡ Total	% mortality	95% confidence interval, % Upper	% Lower
20-A-47	Yes	No	21/62	33.9	22.1	45.7
" PI	No	Yes	18/60	30	18.4	41.0
20-A-67	"	"	35/51	68.6	55.9	81.4

* Data pooled from 5 exp. † See Table I. ‡ Animals starved 4 days and given calcium carbonate *per os* before and opium intraper. following oral challenge with approximately 3×10^7 cells.

to infect mice in order to bring about a fatal infection in guinea pigs. Assuming—and we agree that it is a large assumption—that the pathogenesis of the infection as it occurs in guinea pigs more closely resembles the human than does the mouse infection, it would necessarily follow that the mouse test cannot measure all of those factors which may be of potential importance in establishing the disease in humans. If these factors happen to be antigens, their presence or absence might not be detected or evaluated in a mouse protection test. Yet it is this type of test which is now generally used to measure the potency of cholera vaccines. Thus, in this regard, the implications of the present work are obvious.

Summary. Mouse virulence of a purine-requiring strain of *Vibrio comma* is significantly increased if purines are injected at the same time as the challenge suspension. A purine-independent mutant isolated from this strain was significantly more virulent for mice than the parent culture. A concomitant increase in ability to fatally infect guinea pigs by the oral route was not noted in the mutant strain.

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Received November 20, 1959. P.S.E.B.M., 1960, v103.

TAMe Esterase Activity of Blood Thrombokinase after Repeated Electrophoretic Fractionations.* (25519)

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In recent studies thrombokinase was prepared from bovine plasma(1) and purified further by continuous electrophoresis which separated most of the contaminating thrombin. TAMe esterase appeared in 2 peaks, corresponding respectively to thrombin and thrombokinase peaks(2). Material from the thrombokinase peak has now been subjected to repeated electrophoretic fractionations; and there has continued to be a close correspondence between esterase and kinase activities.

Methods and materials. Continuous paper electrophoresis was performed in a refrigerated Spinco Model CP cell. Buffer: veronal, pH 8.6; ionic strength 0.02. Current, 50 ma. Protein was estimated by the method of Lowry *et al.*(3), with crystallized bovine albumin as standard; and values are subject to limitations noted by them. Thrombokinase was assayed by its capacity to activate prothrombin in presence of cephalin, calcium and bovine barium carbonate serum(1). Activity was expressed in terms of a working standard which has been stored at –23°C and used at intervals for 6 years. A value of 10 indicated that the fraction showed 10 times as much activity/ml as did the standard kinase solution. Esterase activity on tosylarginine methyl ester (TAMe) was determined by the method of Sherry and Troll(4). Thrombin was estimated as described(1), with a dry sample of NIH thrombin as the ultimate standard. Beginning with thrombokinase obtained in a yield of 1.2 mg/liter of plasma, a series of

* This investigation supported by Research Grant from Nat. Heart Inst., P.H.S.

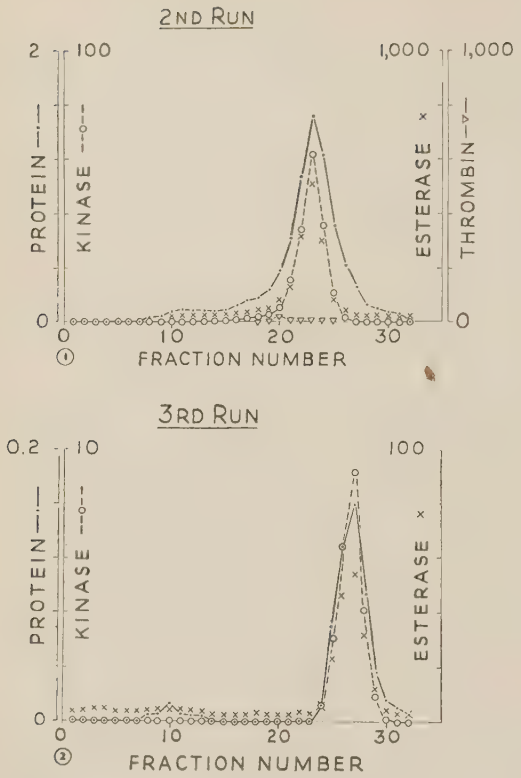

FIG. 1. Continuous flow paper electrophoresis of thrombokinase prepared from bovine plasma. 2nd run. Protein, mg/ml. Kinase, activity/ml, relative to working standard. Esterase, TAMe units/ml. Thrombin, NIH units/ml.

FIG. 2. Continuous flow paper electrophoresis of thrombokinase. 3rd run. Units of protein, kinase and esterase same as for Fig. 1. Input and fractions were less concentrated than those of 2nd run; and scale has been adjusted accordingly.

first electrophoretic runs was made, essentially as described(2). The best thrombokinase fractions were stored at –23°C until the product from 738 liters of plasma had been collected. When these were thawed and pooled, the volume was 455 ml. After addition of 455 ml cold distilled water and 228 ml 0.1 M acetic acid, the pH was 4.8. The resulting precipitate was collected by centrifugation at 4°C and dissolved in cold veronal buffer to make 11.4 ml. This solution was then subjected to the 2nd electrophoretic run.

Results. Thrombokinase appeared in a single peak (Fig. 1) and closely accompanied by esterase activity. The small amount of thrombin reached its highest level at fraction 20, and was quite inadequate to account for the esterase. Protein was spread out more broadly than kinase, and this revealed the presence of a significant amount of protein impurity.

For the 5 best fractions of Fig. 1, average specific activity for esterase was 246 TAMe units/mg protein; average ratio of kinase activity to protein was 28.2. Average ratio of esterase to kinase was 8.7.

However, the best fraction, No. 23, had 339 TAMe units/mg protein, and a kinase to protein ratio of 40.4. Fraction 23 was then subjected to isoelectric precipitation. The precipitate was dissolved in buffer to make 8.0 ml, used as the input for the 3rd run.

The 3rd run (Fig. 2) showed improvement, but a small amount of protein impurity was still evident in Fractions 8-13 and 30-32. No thrombin was detected by usual tests. Fraction 27 was assayed for kinase at protein concentration of 0.0016 μg/ml and a dilution of 1/100,000. This dilution was approximately equivalent to diluting fraction 27 to the volume of parent plasma, 738 liters.

For the 5 best fractions of Fig. 2, average specific activity for esterase was 337 TAMe units/mg protein; average ratio of kinase to protein was 44.7. Average ratio of esterase to kinase was 7.9, the same as before, within limits of error.

Capacity of purified thrombokinase to activate prothrombin in presence of 0.01 M oxalate was verified for the 3 best fractions of the 3rd run.

Discussion. These results add further weight to the hypothesis that thrombokinase is a trypsin-like enzyme. TAMe is a good substrate for trypsin, and it now appears to be a substrate for thrombokinase. Sherry *et al.*(5) anticipated that TAMe might be a substrate for the natural activator of prothrombin, and concluded that more purified concentrates would be necessary to test the hypothesis. Arscott *et al.*(6) found that thromboplastin generating systems prepared with plasma and serum components exhibited TAMe esterase characteristics. The present thrombokinase may well represent the prime, specific factor in their systems.

The defining property of thrombokinase is

its capacity to activate prothrombin. In further characterization, other properties have been described for thrombokinase prepared from plasma(7). It was not sedimented appreciably in 2 hours at 85,000 g, and thus differed from certain easily sedimentable thromboplastins. It converted prothrombin to thrombin in presence of excess oxalate, but was much more effective when accompanied by platelets (or cephalin) plus calcium. Activation of prothrombin by thrombokinase was suppressed by soy bean trypsin inhibitor.

It is now added that purified thrombokinase has shown esterase activity of the order of 337 TAMe units/mg protein. This is more than half that reported for highly purified thrombin and several times that reported for highly purified plasmin(4). No conclusions are drawn regarding relative purity of these preparations, since degree of TAMe esterase activity characteristically varies from one enzyme to another.

These properties of thrombokinase may facilitate comparison with other preparations of clotting factors, purified by electrophoresis (8,9).

Summary. Thrombokinase, purified from 738 liters of bovine plasma, was subjected to repeated fractionations by continuous flow paper electrophoresis. This increased the specific activity of kinase and led to a 3rd electrophoretic run which placed almost all the protein in a single peak. Kinase activity was closely accompanied by TAMe esterase activity through successive stages of electrophoretic fractionation. The kinase fractions of the 3rd run showed esterase activity of the order of 337 TAMe units/mg protein. The results are in accord with the view that TAMe is a substrate for thrombokinase and that thrombokinase is a trypsin-like enzyme.

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Received November 23, 1959. P.S.E.B.M., 1960, v103.

Effect of Glucose and Insulin on Magnesium Metabolism in Rabbits. A Study with Mg^{28}.*† (25520)

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The factors regulating metabolism of magnesium are not well understood, although previous studies have suggested that it may be related to the metabolism of carbohydrate and insulin(1,2). Tissue distribution of radioactive magnesium (Mg^{28}) following its intravenous administration into normal rabbits has been described(3). Our purpose was to determine whether administration of glucose and insulin would alter the exchange of Mg^{28} in rabbit tissues.

Material and methods. Normal domestic adult rabbits of both sexes, with initial body weights between 2 and 4 kg were kept in individual stainless steel metabolism cages and fed a stock diet of compressed pellets. Water was given without restriction. Commercial zinc *insulin* crystals (USP), 40 units/ml, and a sterile 5% solution of *dextrose* in distilled water were used. Mg^{28} was received as $MgCl_2$

* Supported by contract between Univ. of Colorado and U. S. Atomic Energy Comm. and grant-in-aid from Am. Heart Assn. and Colorado Heart Assn.
† Mg^{28} was supplied by Brookhaven National Lab.

in concentrated HCl. 200 μc were contained in 25 to 30 meq of stable magnesium. The material was neutralized with 1 N NaOH and then diluted in physiologic saline solution to contain 0.2 meq of Mg/ml. *Serum* and *urine magnesium* determinations were performed by modification of the molybdivanadate method for phosphate(4). Tissue magnesium determinations were performed by the method of Stutzman(5). Radioactivity assay. Samples of plasma and tissues were assayed for gamma ray activity with a scintillation counter. A total of 10,000 counts were made on each sample. All determinations were corrected for physical decay of the isotope. *Plan of experiments.* A tracer dose of Mg^{28} contained in 1 to 2 meq of stable magnesium was injected intravenously into each animal, and followed by a dose of insulin, glucose, or both. Blood was collected by cardiac puncture or from marginal ear vein into heparinized test tubes. After animal was killed by air embolism, radioactivity content of various tissues was compared with that of plasma obtained at time of death. Chemical determinations for stable magnesium were performed on same samples of tissues that had been assayed for radioactivity. Relative activity of tissue was calculated as follows:

$$\text{Relative activity} = \frac{\text{cpm/g (wet wt) of tissue}}{\text{cpm/ml of plasma}}.$$

In a preliminary study, 6 rabbits which had received the tracer dose of tagged magnesium were each given, by injection into interscapular region, 5 units of insulin/kg of body weight. All animals began to have convulsions within 1 to 1½ hr. Infusion of 5 to 10 ml of a 5% solution of dextrose in water stopped the convulsions. Injections of dextrose were subsequently given at intervals of 30 to 60 minutes as animals appeared overexcited. Animals were killed in pairs at intervals of 2, 4 and 6 hr after injection of insulin and the following tissues were analyzed for radioactivity content: skin, heart, liver, muscle, bone, appendix, kidney and adrenal. When results of these studies were compared with those obtained in normal rabbits which had received tagged magnesium only(3), all animals given insulin and dextrose showed an increase in relative activity of all tissues obtained; relative activities were highest, however, at 4 hr. For this reason, tissues studied in the definitive experiment were obtained 4 hr after initial injection. In this experiment, observations were made on 32 rabbits divided into 4 groups of 8. Animals in *Group A* received only tracer dose of Mg^{28} in 1 to 2 meq of *magnesium.* In *Group B,* all 8 rabbits were given, in addition to tagged *magnesium, insulin* and *glucose.* Four animals in this group were given a single interscapular injection of insulin, 5 units/kg of body weight, followed by 3 doses of dextrose, each 5 ml of 5% solution in water, administered interscapularly at hourly intervals. In the other 4 rabbits, 2 units of insulin/kg were given intravenously, followed immediately by one intravenous injection of 10 ml of 5% dextrose in water. Four rabbits in *Group C* were given, in addition to tagged *magnesium,* an interscapular injection of *insulin,* 5 units/kg body weight, and 4 others were given intravenous injection of *insulin,* 2 units/kg. Besides the tagged *magnesium,* each animal in *Group D* received *dextrose* parenterally. Four were given 10 ml of a 5% solution of dextrose in water intravenously a few minutes after injection of magnesium. Four others were given 3 interscapular injections, each containing 5 ml of 5% dextrose in water, at intervals of one hr.

Results. Group A. Relative radioactivity of the various tissues studied is given in Table I, and magnesium content of tissues is summarized in Table II. *Group B.* Administration of insulin and dextrose resulted in a significant increase in relative radioactivity of all tissues studied. No significant change, however, was found in concentrations of blood glucose or serum magnesium. Significant increases occurred in concentrations of stable magnesium in the heart and the skeletal muscle. *Group C.* Injection of glucose resulted in significant increases in blood glucose concentration and relative radioactivity of the heart. Magnesium content of heart and muscle was significantly elevated. *Group D.* Following administration of insulin alone, the sole change was a significant increase in magnesium content of the heart.

TABLE I. Effect of Insulin and Glucose on Relative Mg^{28} Activity in the Rabbit.

Tissue		Mg (control)	Animals inj. with Mg, insulin & dextrose	Mg & dextrose	Mg & insulin
Bone	$\bar{x}$	12.90	22.10*	18.10	40.50
	$s_{\bar{x}}$	1.13	2.17	1.65	18.10
Kidney	$\bar{x}$	11.00	14.60*	13.20	22.20
	$s_{\bar{x}}$	.60	.94	1.31	6.95
Heart	$\bar{x}$	5.90	12.60*	11.20*	19.40
	$s_{\bar{x}}$	.99	.37	.72	6.05
Liver	$\bar{x}$	5.10	9.80*	7.40	15.40
	$s_{\bar{x}}$	.42	.44	1.25	4.82
Appendix	$\bar{x}$	3.90†	9.90*	7.93	12.78
	$s_{\bar{x}}$	.23	.87	.85	2.57
Skin	$\bar{x}$	1.25	2.34*	2.06	3.56
	$s_{\bar{x}}$	.14	.15	.42	.87
Muscle	$\bar{x}$	.50	1.24*	.89	2.30
	$s_{\bar{x}}$	.10	.14	.09	.82
Serum Mg‡	$\bar{x}$	1.81	1.70	2.03	2.09
	$s_{\bar{x}}$	.33	.09	.09	.08
Blood glucose‖	$\bar{x}$	82.3	69.9	97.1 *	§
	$s_{\bar{x}}$	3.93	4.85	2.28	

$\bar{x}$ = mean value expressed as relative activity, based on data from 8 rabbits.
$s_{\bar{x}}$ = stand. error of mean.
* Significant difference when compared with mean control value. P = less than 0.01.
† Mean of 4 animals.
‡ meq/l.
§ Not done.
‖ mg/100 ml.

Comments. Rate of uptake of Mg^{28} by bone appears to vary with the species studied; Mg^{28} is concentrated slowly in bones of human beings and dogs(6), whereas in the rabbit Mg^{28} is absorbed more rapidly by bone than by any other tissue studied. The next highest relative activities were found in kidneys, heart and liver, while skeletal muscle showed slowest uptake. Magnesium content of bone (300 meq/kg) was at least 13 times higher than that of muscle (22 meq/kg), the tissue with the next highest magnesium content.

Previous studies on rabbits have shown that parenteral administration of magnesium may cause a temporary hyperglycemia or hypoglycemia, depending upon dosage given(2). Intravenous injection of small amounts of magnesium decreases rate of removal of glucose from the blood stream, probably because of impaired peripheral utilization, accelerated glycolysis, or both. Intravenous administration of insulin may result in temporary increase in serum magnesium concentration, followed by a decrease(1). Glucose infusions have been reported to decrease serum magnesium concentrations(5). Insulin and magnesium are said to have a synergistic effect (7).

In the present study, although low specific activity of the isotope made it necessary to subject the rabbits to a slight magnesium load, no significant change in serum magnesium was found when values at 4 hours were compared with serum magnesium concentrations previously obtained in uninjected rabbits(3). While simultaneous injection of magnesium, insulin and glucose did not alter significantly serum magnesium concentration or blood glucose level, it did increase uptake of Mg^{28} in all tissues studied. Since magnesium content of bone, liver and skin did not increase significantly, the increase in Mg^{28} uptake by these tissues is probably due to more rapid exchange of Mg^{28} between their intracellular and extracellular compartments. In skeletal muscle and heart, on the other hand, there was a significant increase in magnesium content as well as in the Mg^{28} relative activity. In these 2 locations, therefore, it is concluded

TABLE II. Effect of Insulin and Glucose on Magnesium Content of Rabbit Tissues.

Tissue		Mg (control)	Animals inj. with Mg, insulin & dextrose	Mg & dextrose	Mg & insulin
Bone	$\bar{x}$	299.5	292.6	309.7	334.4
	$s_{\bar{x}}$	8.92	21.30	6.07	17.70
Muscle	$\bar{x}$	21.5	28.6*	29.8*	20.5
	$s_{\bar{x}}$	.49	1.46	1.17	1.57
Heart	$\bar{x}$	13.0	23.5*	25.9*	16.3*
	$s_{\bar{x}}$	.40	2.21	1.93	.39
Liver	$\bar{x}$	12.4	14.2	13.4	14.6
	$s_{\bar{x}}$	.50	.40	.35	.67
Kidney	$\bar{x}$	12.2	†	12.3	12.0
	$s_{\bar{x}}$	.65		.76	.73
Skin	$\bar{x}$	6.4	5.6	5.2	5.1
	$s_{\bar{x}}$	.26	.31	.37	.48

$\bar{x}$ = mean value expressed as meq of Mg/kg (wet wt) of tissue, based on data from 8 rabbits.
$s_{\bar{x}}$ = stand. error of mean.
* Significant difference when compared with mean control value. P = less than 0.01.
† Not determined.

that actual deposition of increased amounts of magnesium had occurred.

Less striking changes were observed when either insulin or dextrose alone was given following injection of magnesium. It is of interest that magnesium content of both heart and skeletal muscles was increased by administration of dextrose and magnesium, while the sole significant increase in magnesium content following injection of insulin and magnesium occurred in the heart. The combination of insulin and magnesium resulted in the highest mean relative Mg^{28} activities observed in the 4 groups, but the range of values was so wide that no statistically significant differences were elicited.

The biochemical mechanisms whereby dextrose and insulin accelerate Mg^{28} turnover in the tissue are obscure. Animals in this study were subjected to a slight magnesium load. Previous studies indicated that magnesium increases glycogenesis in liver slices(8) and rat-heart slices(9), activates phosphatases from kidneys, intestinal mucosa, liver, bone and plasma, and increases activity of ATP(10). Although it is not known at the present time whether these enzymatic processes are involved in turnover of magnesium, it is of interest that these are the very tissues which in this study showed increased Mg^{28} activity in the presence of insulin and glucose.

Taken as a whole, the observations made in this study and previous studies suggest that metabolism of magnesium is intimately related to that of carbohydrate and that the turnover of magnesium in tissues is regulated by insulin and dextrose. The relationship between metabolism of magnesium and that of other intracellular electrolytes, such as potassium, remains to be studied.

Summary. In rabbits which had received by intravenous injection 1 to 2 meq of magnesium tagged with Mg^{28}, parenteral administration of insulin and dextrose significantly accelerated Mg^{28} uptake in bone, kidney, heart, liver, appendix, skin and skeletal muscle. Tissue magnesium content was increased in heart and skeletal muscle. Less striking changes were noted when insulin or dextrose alone was given following injection of magnesium. Magnesium metabolism appears intimately related to that of carbohydrate and insulin.

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Received November 25, 1959. P.S.E.B.M., 1960, v103.

Effect of ACTH on Regeneration of Adrenal Cortex Following Autografting in Hyophysectomized Rats.*† (25521)

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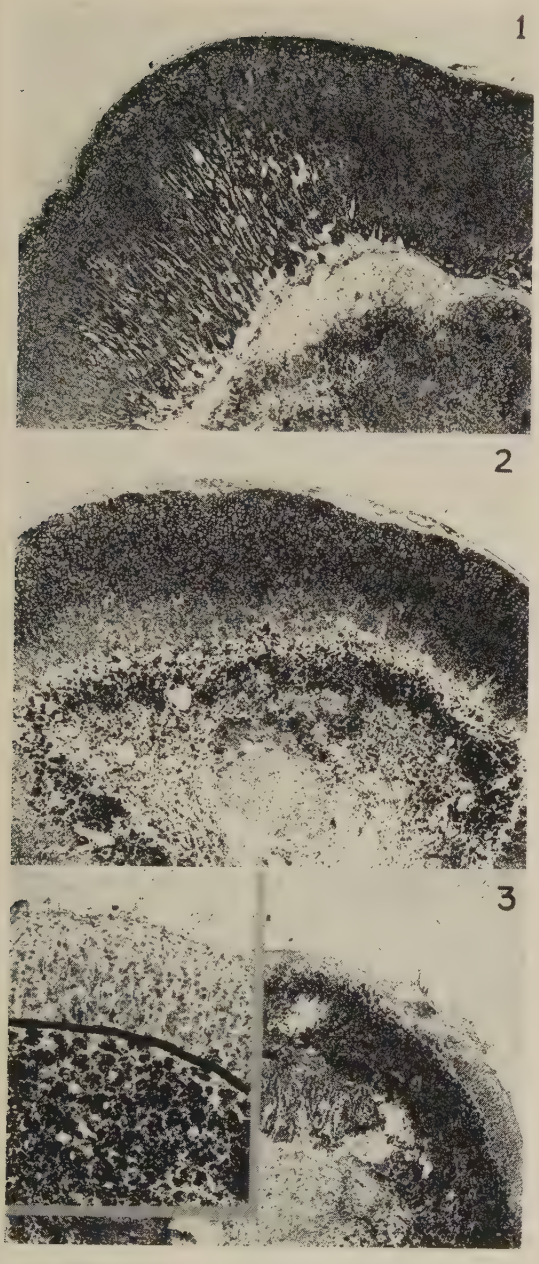
Although adrenal cortical tissue may survive autogenous transplantation in the hypophysectomized rat, regeneration is less than in rats having intact pituitary glands. ACTH treatment of hypophysectomized rats did stimulate regeneration of cortical tissue in

* This research was supported by USPHS grant.

† Grateful acknowledgement is made of assistance of Dr. George F. Wilgram in fixing and microscopic examination of tissues.



Frozen sections of 3 adrenal glands one mo following autotransplantation (oil-red-O-stain) × 25.

FIG. 1. Regeneration of adrenal cortex in a non-hypophysectomized rat.

FIG. 2. Regeneration of adrenal cortex in a hypophysectomized rat inj. with ACTH.

FIG. 3. Regeneration of the adrenal cortex in untreated hypophysectomized rat. *Insert* — High power photograph illustrating an outer atrophic cortex and an inner zone of lipid-laden debris.

adrenal autografts, but extent of regneration was less than occurred in non-hypophysectomized rats.

Methods. Female Sprague-Dawley rats, approximately 3 months old and weighing 185-235 g, were maintained on Rockland Rat diet. Operations were done as described by Ingle(1). The gland was excised, freed of fat, and attached by a fine silk suture to the corresponding ovary. Hypophysectomy was by the usual parapharyngeal approach. All rats received 5,000 units of penicillin and 5 mg of streptomycin on day of operation only. Long-acting ACTH in gelatin (Depo-ACTH, Upjohn, 40 units per cc) was given twice daily by subcutaneous injection. Adrenal cortex extract in saline (1 u per cc, Upjohn) was given in amounts of 1 cc per rat/day for 7 days following hypophysectomy. One month after transplantation, adrenal grafts were removed from the ovaries and weighed, and frozen sections prepared with oil-red-O-stain. Extent of regeneration was rated on the basis of width of the fasciculata zone and appearance of cells, on a scale of 0 to 5; 2.5 approximated the normal adrenal cortex of an unoperated rat. The amount of lipid present was also rated on scale of 0 to 5, 2.5 approximating amount of lipid in normal cortex of the rat. After removal of the grafts, the animals were observed for signs of adrenal insufficiency. At necropsy a careful search was made for accessory adrenal tissue and for remnants of pituitary tissue. When such were found, the animal was discarded from the series.

Results. In non-hypophysectomized rats, adrenal autografts showed good regeneration (Fig. 1, Table I) with more lipid deposition than is seen in the normal adrenal cortex. Observations on regeneration were as described by Ingle and Higgins(2). The medulla of the adrenal does not survive.

Regeneration of cortical tissue was observed in 2 groups of hypophysectomized rats, one given 10 and one given 20 units of ACTH daily (Fig. 2, Table I). Since weights and appearance of grafts were not significantly different between one group and the other, the data are averaged together (Table I). Each of the 3 zones of the cortex was fairly well developed although narrower and possessing less

TABLE I. Mean Weights of Adrenal Glands and Microscopic Evaluation of Regeneration and Lipid Deposition in Adrenal Glands One Month after Transplantation.

Group	Procedure	No. of rats	Adrenal wt* (mg)	Adrenal wt* (mg/100 g body wt)	Adrenal cortex† Width (mm)	Adrenal cortex† Regeneration	Adrenal cortex† Lipid deposition
A	No hypophysectomy; no ACTH given	14	29.6 ± 1.8	10.3 ± .6	1.15	3.0 ± .4	3.0 ± .3
B	Hypophysectomy; 5 and 10 units ACTH given twice daily	17	14.8 ± 1.9	7.3 ± .7	.77	2.3 ± .8	2.7 ± .3
C	Hypophysectomy; no ACTH given	10	6.8 ± .9	3.5 ± .5	.18	1.8 ± .7	.6 ± .8

* Mean ± S.E.

† Regeneration and lipid deposition are rated on scale of 0 to 5, a value of 2.5 being comparable to appearance in unoperated normal rats.

lipid than autografts in non-hypophysectomized rats. The cells were vacuolated and composed of light cytoplasm, suggesting possible overstimulation. Autografts in hypophysectomized rats without ACTH showed some viable lipid-laden cortical tissue, but most of each graft was necrotic and regeneration remained minimal (Fig. 3, Table I).

Discussion. The results show that regeneration of an apparently well organized adrenal cortex can be stimulated by ACTH alone in the hypophysectomized rat. Extent of regeneration is less than occurs in presence of the anterior pituitary. Amounts of long-acting ACTH used in these experiments were very large. It is possible that the response of the adrenal graft to exogenous ACTH could be normalized if the ACTH were given continuously. It is also possible that some hypophyseal hormone other than ACTH plays a role in regeneration of adrenal cortical grafts.

Summary. Large doses of ACTH stimulated autografts of adrenal glands in hypophysectomized rats to regenerate cortical tissue. Extent of regeneration was less than occurs in the non-hypophysectomized rat.

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Received November 23, 1959. P.S.E.B.M., 1960, v103.

Colorimetric Determination of Amino Acids in Presence of a Peptide. (25522)

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In investigating the splitting of peptides by peptidases, there has been need for a simple, quick photometric method to determine quantitatively the amino acids present after hydrolysis. The familiar Moore and Stein ninhydrin method(1) for determination of amino acids is not applicable as color intensity from peptides, on a molar basis, is nearly the same as that from amino acids. Most investigators use a titration method(2) for estimation of liberated carboxyl groups. In the present investigation, it was observed that when pH of the buffer in the ninhydrin reagent was 4.0 instead of 5.5, and development of ninhydrin color was at 60°C instead of 100°C, as used by Moore and Stein, amino acids gave the usual amount of color while peptides remained almost colorless. The glycyl peptides, which gave about 50% as much color as amino acids, were an exception to this. The hydrolysis of peptides can be readily followed, when these modifications of the Moore and Stein procedure are used to determine ninhydrin-reacting substances, since the color developed is

due almost exclusively to the amino acids liberated during the hydrolysis.

Materials and methods. Ninhydrin reagent. 0.5 g of ninhydrin and 25 mg of hydrindantin were dissolved in 18.75 ml of methyl cellosolve (peroxide free). This solution was diluted to 25 ml with 0.05 *M* potassium acid phthalate, pH 4.0, and was made up fresh for immediate use because this reagent was not stable for more than 1-2 hours. *Standard Solutions of Amino Acids and Peptides.* Amino acid solutions were made up at 3.0 *mM* in 0.025 *M* tris (hydroxymethyl) aminomethane buffer at pH 8.0. Peptide solutions were made up at 1.5 *mM* in the same buffer. *Determination of standard curves.* A standard curve showing change of optical density with concentration was prepared for a dipeptide and its hydrolysis products as follows: A mixture of a dipeptide and its constituent amino acids were prepared to simulate a range of products present after enzymatic hydrolysis of the dipeptide. To a series of 5 test tubes were added 0, 0.17, 0.33, 0.67, and 1.0 ml of a solution prepared by mixing equal volumes of 2 standard 3.0 *mM* amino acid solutions, each amino acid then being present at a 1.5 *mM* concentration. To the same series of test tubes were added 1.0, 0.83, 0.67, 0.33, and 0 ml of a standard 1.5 *mM* solution of the dipeptide, making a total volume of 1 ml per tube. One-tenth ml aliquots were removed from each tube and placed in another series of 5 tubes, which contained 0, 0.025, 0.05, 0.10, and 0.15 μmole of each amino acid plus 0.15, 0.125, 0.10, 0.05, and 0 μmole respectively of dipeptide. The blank contained 0.1 ml of 0.025 *M* tris buffer, at pH 8.0. One ml of ninhydrin reagent was added to each tube, which were then immersed 30 minutes in a 60°C water bath to obtain maximum color development. The tubes were removed, cooled, and diluted to 10 ml with 50% ethanol. After standing 5 minutes, the ninhydrin color was read at 575 mμ in a Lumetron photometer.

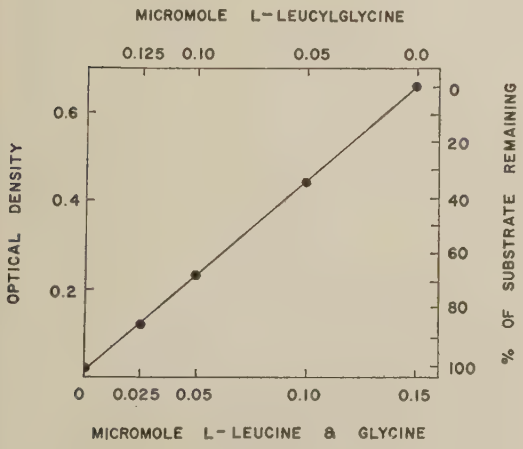

FIG. 1.

TABLE I. Color Yields on a Molar Basis from Amino Acids and Peptides Relative to Glycine.

Compound	Color yield	Compound	Color yield
Glycine	1.00	Leucylglycine	.03
Leucine	.78	Leucylalanine	.09
Alanine	.97	Leucylphenylalanine	.03
Valine	.97	Alanylglycine	.04
Lysine	.98	Phenylalanylglycine	.04
Arginine	.90	Valylglycine	.00
Histidine	.75	Lysylglycine	.03
Phenylalanine	.17	Arginylglycine	.09
Glycylglycine	.57	Leucylglycylglycine	.12
Glycylalanine	.54	Leucinamide	.12
Glycylleucine	.40		

Results. Fig. 1 was used as a reference standard for determining enzymatic hydrolysis products of L-leucylglycine. Similar reference curves (not shown) were prepared for analysis of reaction products from other peptides used as substrates. Other buffers, such as citrate and acetate, were employed in the ninhydrin reagent. Potassium acid phthalate, although having the least buffering capacity, was chosen because it gave the most linear curve over a range of zero to 0.30 μmole of amino acids plotted against optical density.

Table I demonstrates color yields on a molar basis of amino acids and peptides tested, relative to glycine value of unity. These were determined by adding 0.1 ml of a 1.5 *mM* solution of an amino acid or peptide in 0.025 *M* tris buffer to one ml of ninhydrin reagent. Procedure for development of color was carried out as described above. Color yields as listed in Table I are ratios of optical densities of amino acids or peptides to optical density of glycine. Color yields from peptides seem to be related to their structures. Glycyl peptides give considerably more color than those peptides containing a free amino group

attached to a carbon atom with a side chain. For example, glycylalanine yields more than 10 times as much color on a molar basis as alanylglycine.

Summary. A photometric method for determination of free amino acids in presence of a peptide has been described. It is applicable to estimation of free amino acids present after hydrolysis of peptides by peptidases.

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Received November 30, 1959. P.S.E.B.M., 1960, v60.

Effect of Subconvulsive Audiogenic Stress in Mice on Turpentine Induced Inflammation.* (25523)

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In previous studies we explored certain mechanisms involved in mobilization of organism's defenses against injury. Various procedures were employed, including effect of cortisone(1,2), of antireticulocytotoxic serum (ACS)(3), of somatotrophic hormone (STH) (4), and audiogenic stress(5) upon wound healing. Results in each instance suggested that delay or inhibition of wound healing probably relates to a number of common underlying mechanisms. Such a concept would be forced to recognize that stress may well be a major factor capable of affecting such basic physiologic adaptive processes. It was shown that mice subjected to audiogenic stress on 10 consecutive days have definitely impaired capacity for producing good, healthy granulation tissue after subjection to a single surgical wound. The deficit in such tissue repair does not appear to be mediated entirely through the pituitary-adrenal axis(5). There are differences in degree of response in different strains of inbred mice, and sound stimulation is more effective in producing audiogenic seizures when applied at night, rather than during the day. Also, there is a fair degree of parallelism between wound healing effect and nocturnal *vs.* diurnal timing of sound exposure.

Procedures. Because of well known stress effect upon animals of handling or confinement, preliminary studies were carried out to minimize all extraneous factors that might influence the animal other than auditory stimulus. As a result of these tests a small 6′ x 9′ room was constructed with facilities for 70 standard mouse cages, but placed upon specially wired racks with automatic electronic controls to set the interval, time and duration of each bell ringing exposure. This room was tested to determine effective delivery of stress by such bell ringing audiogenic stimulation as to evoke subconvulsive seizures in susceptible strains of mice. Time duration of each exposure ranged from 10 seconds initially to 60 seconds with 5 second increments. Control animals were housed in a comparable room and were handled in identical fashion, except that they were not exposed to bell ringing. Groups of C_{57}BL/6 (seizure resistant) and DBA/1 (seizure susceptible) mice were treated as follows after one week exposure to stress: 1. 20 C_{57}BL/6 and 20 DBA/1 mice given surgically induced wounds. 2. 40 C_{57}BL/6 and 40 DBA/1 mice injected subcutaneously with 0.025 cc turpentine. 3. Control groups of both strains, *not* exposed to stress, but otherwise treated identically. Results revealed a marked delay in wound healing and repression in extent and severity of the inflammatory reaction to turpentine as compared with that observed in the controls. These findings prompted the following to determine the morphologic characteristics of these altered responses and their underlying mechanisms. Groups of 20 mice each of

* Supported in part by grant from Nat. Science Fn. and the South Shore Council of the Waldemar Medical Research Fn.

DBA/1 (seizure susceptible) and A/Jax (seizure resistant) strains, 12 to 14 weeks old and uniformly distributed in respect to sex and weight, were treated as follows: *Group I.* 20 A/Jax and 20 DBA/1 strain mice were exposed to audiogenic stress as described, for 7 consecutive nights, then injected immediately beneath surface epithelium (intracutaneously) with 0.025 cc of turpentine. Stress was applied nightly for 14 nights in accordance with predetermined schedule of sacrifices indicated below. *Group II.* 20 A/Jax and 20 DBA/1 strain mice were exposed for same 7 day pretreatment stress, then injected similarly with 0.025 cc of turpentine, but no further stress was applied. *Group III.* Controls: 20 A/Jax and 20 DBA/1 strain mice were injected with 0.025 cc of turpentine without exposure to stress stimulation. Five animals from each of the 6 groups were sacrificed as follows: 1) 24 hours after turpentine injection, 2) 4 days, 3) 7 days, and 4) 14 days after turpentine injection. Animals were all autopsied and tissues taken from local inflammatory area, from adrenals, liver, spleen and kidneys routinely, and randomly from other organs including lungs, heart and thyroid. Tissues were fixed in 10% formalin, sectioned and stained with hematoxylin and eosin. Slides were numbered by code and studied histologically by one of us (LWS) without previous knowledge of their identity, or treatment given.

Results. Local lesions (*e.g.* site of turpentine injection along with surrounding subcutaneous tissue and overlying epithelium) were graded on a 1-4 scale as follows: *Grade 1,* a relatively small localized cystic lesion containing very few cells and showing only a very narrow limiting zone of inflammatory cellular infiltration; *Grade 2,* the cystic area tends to be somewhat larger, containing small amount of leucocytic exudate and fibrin, limiting inflammatory zone is considerably wider, but there is little or no extension into surrounding tissues; *Grade 3,* the lesion still tends to be localized and cystic but is practically filled with pus, and inflammatory reaction extends widely into surrounding subcutaneous tissues, possibly very early fibroblastic development; *Grade 4,* evidence of fibrous tissue proliferation and epithelization (in the few cases where ulceration was noted), but with marked residual inflammatory cellular infiltration of tissues. Complete healing was not found in any instance.

Any changes observed in various viscera were also graded on a 1-4 scale. In liver and kidney such minor changes as were noted were of a transient, mild, irritative or toxic nature relating principally to various degrees of granular changes of the cytoplasm. In the spleen the gradation was expressed on the basis of follicular hyperplasia and by congestion. There was little or no consistency in these essentially insignificant variations except perhaps that they were most numerous in 24 hour and 4 day animals. For double check, random white blood cell and differential counts were taken from animals in each group. No significant rise in numbers nor alteration in percentage differential count was found, further evidence of the very minor or totally negligible systemic injury produced by audiogenic stress.

Discussion. As preliminary analysis of the statistical data revealed no demonstrable difference in the animals in Groups I and II, they have been grouped together in the final analysis. Nor was there statistically valid difference in the lesions in the 2 strains of mice, although the A/Jax mice appear to show rather less suppression of the inflammatory response during the first 24 hours or even by the fourth day than do the DBA/1 mice. However, these minor differences tend to disappear with the time factor, by the seventh day.

Critical review of the statistical data fails to give a clear-cut answer to the problem of total effect of audiogenic (subconvulsive) stress upon physiologic response of the body to inflammation. On the other hand, the data seem to indicate that stress exerts a repressive effect upon normal inflammatory response, and upon subsequent repair process, much as it was shown to do previously, in causing delay in wound healing experiments. The mechanism of this physiologic response to stress is not clear. The evidence is not adequate to explain it on the basis of adrenal cor-

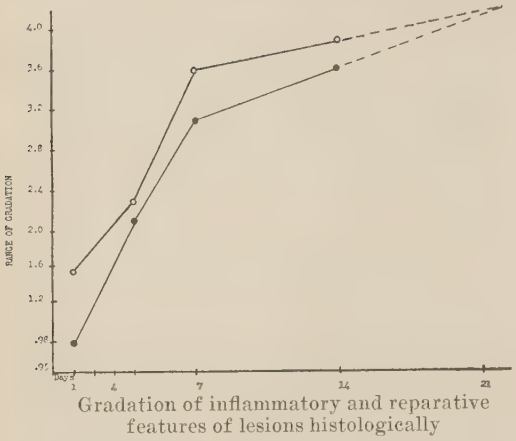

FIG. 1. ●——● experimental; ○——○ control.

tical stimulation, though the effect is comparable to that obtained by the use of cortisone. In this connection, Dr. Robert Henkin, Univ. of California, Los Angeles, has reported complex hormonal stimulation in animals subjected to unpleasant sounds, whereas no such effect is obtained in the presence of pleasant sounds such as music.

Two points have emerged from these experiments: 1) stress of this type does delay and repress to a considerable degree the usual inflammatory reaction to an injurious agent and 2) it likewise represses to a very considerable degree the repair process during the first 10 days to 2 weeks. Ultimately, however, the healing catches up, but in general leaving less residual scarring.

To ascertain whether the age of the mice might have modified the results, the experiments were repeated with 36 young (20 g) DBA/1 strain mice (seizure susceptible). Two or 3 animals from each group were sacrificed on the same 24 hours, 4 day, 7 day and 14 day schedule, their tissues taken and examined microscopically. These supplemental data were somewhat more sharply defined and give added weight to our earlier results. These statistics have been added to the other data and a composite graph (Fig. 1) emphasizes the major differences of experimental *vs.* control response to injection subcutaneously of turpentine following application of subconvulsive audiogenic stress.

Summary and conclusions. 1) A/Jax and C_{57}BL/6 (both seizure resistant) and DBA/1 (seizure susceptible) strain mice were exposed to subconvulsive audiogenic stress for 7 days before, and in some instances up to 14 days after intracutaneous injection with 0.025 cc of turpentine. 2) The effect of pretreatment audiogenic stress resulted in moderate repression of ordinary inflammatory response observed in control animals. This was characterized by a sharp localization of the lesion with only a very narrow limiting zone of inflammatory cells. 3) In addition to relative repression of inflammatory response, delayed repair of injury was observed, with a minimal amount of fibroblastic proliferation during first 10 days or more in the majority of animals. 4) No differences were observed in these reactions in mice subjected to posttreatment stress supplementing the initial pretreatment application of stress. This suggests that the mechanism initiated by such audiogenic stress persists for a considerable time. 5) The data were inadequate to establish the way in which the reaction operates, but a certain parallel may be drawn between inhibition of inflammatory response clinically by use of adrenal cortical steroids to suggest that adrenal cortical stimulation may be involved.

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Received December 2, 1959. P.S.E.B.M., 1960, v103.

Mechanisms of Autoregulation in Isolated Perfused Kidney.* (25524)

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The important role of intrarenal extravascular pressure changes in regulation of blood flow through the kidney has been previously reported(1). The further observation that "intrarenal" vascular resistance (renal artery pressure-tissue pressure/flow) often rises with renal artery pressure elevation suggests that causative factors other than tissue pressure changes are involved in the autoregulation phenomenon(1). Among other possibilities, it has been suggested(1) that extravascular pressures within Bowman's capsule may exceed the general renal tissue pressure, resulting in a fall in glomerular transmural pressure. The effect of glomerular filtration on post-glomerular vascular resistance by virtue of changes in blood viscosity, is also to be considered as an additional factor in elevating renal vascular resistance(2,3). Autoregulation of flow is reported to occur in kidneys perfused with colloidal solutions containing virtually no red blood cells(4,5,6) and is also found in intact kidneys when the blood hematocrit is very low(7). It thus appears that blood viscosity changes cannot be solely responsible for renal flow regulation. Calculations of "intrarenal" resistance (RA-TP/F) (1) have been made in the present study in blood- and dextran-perfused dog kidneys to distinguish between relative effects of tissue pressure and post-glomerular viscosity changes on "overall" vascular resistance. The possible compression effect of Bowman capsule extravascular pressure in providing a fraction of the rise in renal vascular resistance has also been investigated.

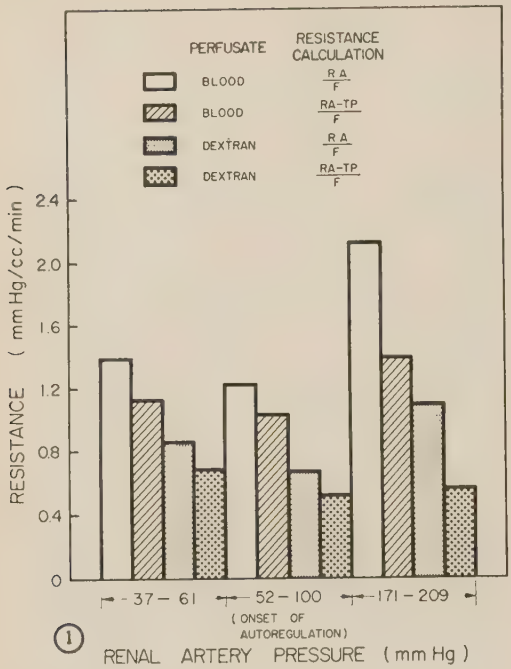
Methods. Kidneys were removed from anesthetized animals without interruption of renal blood flow and perfused at a controlled arterial presure with a pump-lung apparatus as described previously(1). In the first series of experiments isolated kidneys were alternately perfused with 4% dextran and blood as reported previously(6). Renal vein pressures were maintained at zero mm Hg. Tissue pressures and flows were determined as recently described(1,6), and "overall" (RA/F) and "intrarenal" (RA-TP/F) vascular resistances were calculated(1). In a second group of experiments isolated kidneys were perfused with blood during which time the ureters were totally occluded. Tissue and ureteral pressures were simultaneously measured as renal artery pressure was progressively elevated through the autoregulatory range. Ureteral pressures obtained in these studies were assumed to approximate the extravascular pressure in Bowman's capsule, since glomerular filtration is very low(8). All pressures were measured by means of Statham strain gauges and registered on a direct writing four channel Polyviso Sanborn recorder.

Results. Data from blood- and dextran-perfused kidneys are presented in Fig. 1. Mean values for "overall" and "intrarenal" resistances have been obtained from 14 perfusion studies. Vascular resistance calculations have been grouped into 3 renal artery pressure levels: (a) 37-61 mm Hg (prior to onset of autoregulation); (b) 52-100 mm Hg (at onset of autoregulation when all resistances are at lowest values); (c) 171-209 mm Hg (when resistances are at highest values). Although it is seen that "intrarenal" vascular resistance rises somewhat through the autoregulatory range in blood-perfused kidneys, it increases very little during the same period in dextran-perfused kidneys.

Fig. 2 depicts data obtained in a separate study when 3 isolated kidneys were perfused with blood, and ureters were continuously clamped. As renal artery pressure is progressively elevated, both tissue and ureteral pressures rise to varying degrees. Ureteral pressure is observed to increase progressively more

* Research supported by grant from Graduate School, Univ. of Minn.

† Research performed during term of Lederle Medical Faculty Award, 1959-1962.



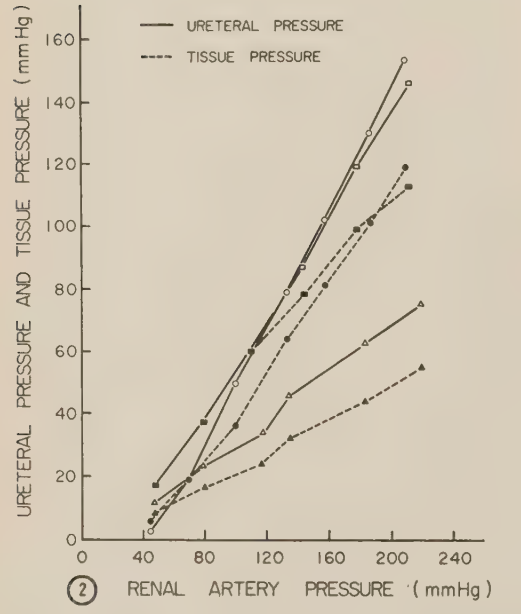


FIG. 1. Mean values of "overall" (RA/F) and "intrarenal" (RA-TP/F) vascular resistance in blood- and dextran-perfused dog kidneys. Resistances are calculated from data obtained following progressive elevation of renal artery pressure.

FIG. 2. Ureteral and tissue pressures obtained in 3 isolated blood-perfused dog kidneys during progressive elevation of renal artery pressure (ureters continuously clamped). Solid and open symbols refer to tissue and ureteral pressures, respectively, with identically-shaped symbols representing each separate kidney perfusion.

rapidly than tissue pressure as arterial pressure is elevated.

The 3 factors considered responsible for autoregulation in the isolated perfused kidney are illustrated in Fig. 3 and approximate fractional roles of each have been assigned. The increment in resistance due to excess of Bowman capsule extravascular pressure over general tissue pressure has been designated as "Bowman capsule pressure."

Discussion. The present study was designed to evaluate the relative effects of factors considered responsible for the autoregulation phenomenon. Findings suggest that blood flow regulation in the isolated perfused kidney is brought about by (a) the combined effects of extravascular pressures within and without Bowman's capsule and (b) changes in blood viscosity resulting from glomerular filtration. The latter factor is considered to be extremely variable, depending on intrarenal hematocrit and glomerular filtration rate(2, 10). Although other factors may perform additional roles in increasing resistance, in view of the present data, their effects need not be included to account for the phenomenon of autoregulation.

In contrast to the observations of Malvin and Wilde(8), renal blood flow was markedly decreased following occlusion of the ureter, as was also reported by Winton(9). Autoregulation of blood flow occurred to a high degree during continuous occlusion of the ure-

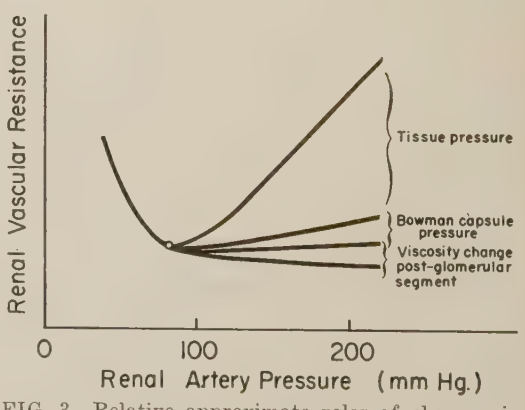


FIG. 3. Relative approximate roles of changes in tissue pressure, Bowman capsule extravascular pressure and post-glomerular blood viscosity on renal vascular resistance in the isolated perfused dog kidney.

ter, although estimations of pre-glomerular resistance showed no rise throughout the autoregulatory period. The observation that ureteral pressure is greater than tissue pressure suggests that Bowman capsule extravascular pressure is an effective compressing force on the renal vascular bed. These data also suggest that tubules possess considerable structural rigidity.

Mechanisms of autoregulation, according to the present study, appear to operate primarily in association with glomerular filtration and also extend their effects through the post-glomerular segment. The "clamping" effect of extravascular pressure on intra-parenchymal arterial vessels has not been evaluated.

Summary. The present investigation has been concerned with mechanisms explaining autoregulation of renal blood flow. Data from experiments on the isolated perfused dog kidney suggest that as renal artery pressure is elevated, blood flow regulation is brought about by (a) combined effects of increased tissue pressure and Bowman capsule extravascular pressure, and (b) increases in post-glomerular viscosity resulting from glomerular filtration.

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Received December 3, 1959. P.S.E.B.M., 1960, v103.

Influence of Sodium Thiosulfate on Reducing Capacity of Human Erythrocytes *in vivo*.* (25525)

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Ability of the red blood cell to reduce iodine is, to a great extent, a function of its glutathione content(1). We observed that, during intravenous infusion of sodium thiosulfate, there occurs a diminution in reducing capacity of red blood cells. We established an index determined by the ratio between this diminution and amount of sodium thiosulfate infused. Erythrocytes of normal women had a reductivity index (R.I.) which was more than double that of normal men.

Method. One hundred ml of 10% $Na_2S_2O_3 \cdot 5\ H_2O$ was infused intravenously as described by Cardozo and Edelman(2). Average duration of infusion was 10 minutes. Venous blood was withdrawn in heparinized syringes at 0, 25, 35, 45, and 60 minutes after beginning of infusion. Each blood specimen was divided into 2 portions. The first part was used for iodimetric titration of whole blood, the second portion for iodimetric titration of serum(3). Volume of iodine used for each titration is employed to determine reducing capacity of the sample at each time interval and is measured in terms of arbitrarily devised reductivity units (r.u.). Ten r.u. are equal to that volume of N/1000 iodine standard required to titrate 0.5 mg of sodium thiosulfate standard. The N/1000 iodine solution was prepared from N/10 iodine(4) by dilution. One g KI/100 ml was then added to stabilize final solution as recommended by

* Aided by grant from Muscular Dystrophy Assn.
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Brun(3). The N/1000 iodine solution was standardized against N/400 sodium thiosulfate(3), which was stabilized with 2-3 g of borax/liter. Fresh standards were prepared monthly. Whole blood reducing capacity at each time interval was measured as follows: A 1:10 dilution of original blood sample was prepared by laking 1 ml of heparinized blood in 5 ml of distilled water with gentle agitation. The proteins were then precipitated with 4 ml of freshly prepared Folin-Wu reagent (1 vol. 10% sodium tungstate: 1 vol. ⅔ N sulfuric acid: 2 vol. distilled water). After precipitation, specimens were centrifuged for 7-10 minutes at 2500 rpm, and the protein-free supernatant was filtered through Whatman #12 paper. One ml of protein-free filtrate was then titrated by direct iodimetry at room temperature, using 1% starch reagent to establish the end-point. Just prior to each titration, 1 ml of 2 N HCl was added to the titration tube. Reducing capacity of serum at each interval was measured by making a 1:5 dilution of the original sample as follows: heparinized blood was centrifuged 10 minutes at 2500 rpm. One ml of serum was combined with 4 ml of Folin-Wu reagent to precipitate proteins. After precipitation, specimens were centrifuged for 7-10 minutes at 2500 rpm. One ml of protein free supernatant was then titrated by direct iodimetry as described above. All titrations of blood and serum were done in duplicate, subtracting blank values of tubes containing similar volumes of water. No more than 2 hours were permitted to elapse between each venipuncture and final titration of its sample. To calculate red blood cell reducing capacity at a specific time (t) the following formula was used: $R_t = \frac{B_t - (1 - HCT)\,\S S_t}{HCT}$. R_t is reducing capacity of red blood cells/ml rbc. B_t is reducing capacity of whole blood/ml whole blood. S_t is reducing capacity of serum/ml serum. At conclusion of each experiment, R_t was plotted as ordinate on graph paper, and time (min.) plotted as abscissa (Fig. 1). Clear portion represents loss in red blood cell

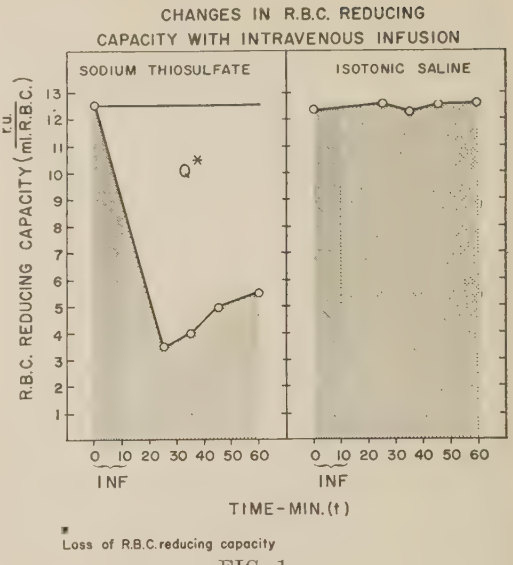

FIG. 1.

reducing capacity in one hour. When this area (Q) is divided by amount of sodium thiosulfate infused, the reductivity index (R.I.) is established.

$$\text{R.I.} = \frac{\text{Area represented by Q in unit-min.}}{\text{Total amount of sodium thiosulfate (g)}}:$$

Amount of thiosulfate infused is calculated by diluting 1 ml aliquot of the 100 ml infusion in 1 liter volumetric flask using borax as preservative and titrating 1 ml of the 1/1000 dilution by iodimetry as described for protein-free filtrates above. Simultaneous calculations were made to determine extracellular fluid volume(2) and to calculate disappearance rate (k) of sodium thiosulfate from plasma according to the formula: $k = \frac{0.693\|}{T\,½}$ where T½ is half life of sodium thiosulfate in plasma. Red blood cell reducing capacity pattern following intravenous infusion of 100 ml of isotonic saline is demonstrated for control purposes. Reductivity indices were established in 14 adult white females and 10 adult white males. In addition, 3 female patients were given 75 mg testosterone propionate daily intramuscularly for 7 days. Indices were measured prior to and following course of testosterone. Two additional male patients were given 5 mg stilbesterol tablets

§ HCT is hematocrit.

‖ Nat. log of 2.

TABLE I. Erythrocyte R.I. in 14 Women.

Age range (yr)	R.I. range	Avg R.I.	S.D.
29-71	68-127	87	17

daily for 1 week. Reductivity indices were measured prior to and following this estrogen. Indices were also determined in 3 boys and 1 girl during puberty, a 25-year-old man with hyperparathyroidism, a 49-year-old man with carcinoma of bladder, and a 50-year-old castrated man with carotid artery occlusion and hemiplegia. A final study was performed by administering testosterone propionate to this castrate in dosage listed above, and control R.I. was compared with post-testosterone R.I.

Results. Adult women (Table I) had an average R.I., 87, which was significantly greater ($p<.001$) than the average R.I., 34, of normal adult men (Table II). Administration of testosterone propionate (Table III)

TABLE II. Erythrocyte R.I. in 10 Men.

Age range (yr)	R.I. range	Avg R.I.	S.D.
30-63	28-42	34	5

resulted in considerable depression of R.I. in 2 of 3 women and in the castrated man. The opposite effect was obtained following administration of stilbestrol to 2 normal men (Table IV). Differences in R.I. could not be attributed to variations in extracellular fluid. Sodium thiosulfate estimation of the extracellular compartment in men averaged 18.8% of total body weight; in women, 19.6% of total body weight. Two patients given 100 mg cortisone acetate orally daily for one week showed 3 liter increases in extracellular fluid but no change in R.I. A third patient, given 100 μg l-triiodothyronine orally daily demonstrated 16% fall in R_o¶ after one week, but no change in R.I. No sex difference was found in (k) rate of disappearance of sodium thiosulfate from plasma which averaged 0.016/min. In 4 patients undergoing pubertal changes, one 14-year-old girl and one 14-year-old boy showed R.I. values of 69 and 34 respectively, consistent with normal adult values. The other R.I.'s are intermediate. High R.I. values were encountered in 2 men with neoplastic disease. Average R_o for women and men were 15 and 17 r.u./ml rbc respectively equivalent to 0.93 and 1.05 mg GSH/ml rbc.

TABLE III. Effect of Testosterone on Erythrocyte R.I.

Age	Sex	Pre testosterone	Post testosterone
80	♀	92	32
54	♀	94	37
64	♀	93	78
50	♂ (castrate)	71	46

TABLE IV. Effect of Stilbestrol on Erythrocyte R.I.

Age	Sex	Pre stilbestrol	Post stilbestrol
38	♂	37	78
41	♂	29	41

Discussion. Reducing capacity of red blood cells under normal conditions is, in large measure, a function of reduced glutathione (GSH) content and does not vary appreciably during one hour, nor does it change following intravenous infusion of isotonic saline (Fig. 1). This capacity falls significantly following intravenous infusion of sodium thiosulfate. The absolute and relative diminution in this capacity differs between sexes in normal adults, resulting in differences in R.I. Since there is no sex difference in erythrocyte mean corpuscular volume(5), size of red blood cell is probably not a factor. Preliminary studies suggest that circulating androgen and estrogen may play a role in response of erythrocyte reducing capacity to intravenously administered sodium thiosulfate. Diminution in this capacity reflects changes in intracellular oxidation-reduction of glutathione favoring the oxidized form. The roles of glucose-6-phosphate dehydrogenase and glutathione reductase have not yet been evaluated in the R.I. phenomenon. It has previously been shown(6,7) that intracellular glyoxalase activity influences glutathione stability, and that interferes with this stability by inhibiting glyoxalase activity. Since arsenate and thiosulfate behave similarly in reduction-oxidase systems, it is conceivable that R.I. may be a function of similar process.

¶ $R_o = R_t$ when $t = 0$

Further investigation is proceding to elucidate the mechanism of the R.I. phenomenon and to see whether it relates to neoplasia.

Summary. Reducing capacity of erythrocytes has been measured. Followng intravenous infusion of sodium thiosulfate, there occurs a significant diminution in this capacity. A reductivity index (R.I.) was established relating diminution in this capacity with amount of sodium thiosulfate infused. R. I. of erythrocytes of women is more than double that of normal men. Preliminary studies suggest that estrogen and androgen may influence R.I.

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Received December 3, 1959. P.S.E.B.M., 1960, v103.

Dietary Vitamin K Requirement of the Rat. (25526)

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Recent reports by Barnes and Fiala(1) and by Mameesh and Johnson(2) have shown that a dietary Vit. K deficiency can be produced in the non-coprophagic rat. Prior to the finding of the role of coprophagy in supplying intestinally synthesized Vit. K to the rat(1,2), it had not been possible to determine the quantitative Vit. K requirement of the rat for maintenance of a normal prothrombin level. The experiments reported herein were conducted to measure the Vit. K requirement of the growing, non-coprophagic, male rat.

Methods. Weanling male albino rats of the Sprague-Dawley strain were randomly divided into groups of 10 rats each. The animals were individually housed in wire-bottomed cages in a temperature-controlled laboratory. Food and water were given *ad libitum.* Daily feed records were kept, and the rats were weighed at weekly intervals. Coprophagy was prevented in all rats by the method of Barnes *et al.*(3). The composition of the basal diet is given in Table I. The diet was mixed every 2 weeks and stored in dark brown glass jars under refrigeration. To reduce bacterial growth, the daily feed refused was discarded and feed dishes and water bottles were washed every other day with hot water and a household detergent. Vit. K_1 (3 - phytyl - 2 - methyl - 1,4 - naphthoquinone, Mann Research Laboratories, Inc., New York) was dissolved in triolein and added to the diet at the time of mixing at predetermined levels. Evidences for inadequate Vit. K intake were: a) hemorrhagic deaths with one or more of the following symptoms ob-

TABLE I. Basal Diet.

Ingredient	g
Sucrose	66.9
Drackett-soy protein (Archer Daniels Midland Co., Cincinnati, O.)	20.0
DL-methionine (Dow Chemical Co., Midland, Mich.)	.5
Triolein	3.0
Vitaminized cerelose*	5.0
Salt mixture No. 446†	4.0
Methyl linoleate urea inclusion compound (25% methyl linoleate, Hormel Foundation, Austin, Minn.)	.6
α-Tocopherol acetate	.012
Vit. A (Nopcay, 250,000 USP units per g, Nopco Chemical Co., Harrison, N. J.)	.008
Vit. D_3 (Super Nopdex 15,000 I units/g, Nopco Chemical Co., Harrison, N. J.)	.013

* Mameesh and Johnson(2). † Spector(5).

TABLE II. Mean 4-Week Gains, Feed Intakes, Incidence of Lethal Hemorrhages and Plasma Prothrombin Times of Groups of 10 Non-Coprophagic Rats Receiving Various Levels of Vit. K_1.

	Vit. K_1 admin. (μg/g diet)	Avg 4-wk gain (g)	Avg 4-wk feed intake (g)	Hemorrhagic deaths	Avg prothrombin time (sec.)
Exp. I	0	103 ±15.6*	279 ±19.5*	5	58.6 ±8.8*
	.2	169 ± 5.3	386 ± 5.8	0	13.2 ± .2
	.4	165 ± 4.3	380 ± 8.6	0	13.8 ± .3
	.6	162 ± 3.3	368 ± 6.7	0	13.9 ± .2
	.8	154 ± 4.8	359 ± 7.8	0	14.2 ± .2
	1.0	159 ± 1.8	379 ± 4.5	0	14.0 ± .1
Exp. II	0	156	366	9	83
	.05	146 ± 4.1	359 ±10.4	2	25.0 ±2.3
	.10	159 ± 3.2	380 ± 6.1	0	14.8 ± .2
	.15	157 ± 3.6	372 ± 5.8	0	13.9 ± .2
	.20	157 ± 3.2	387 ± 6.3	0	13.9 ± .2

* Stand. error of mean.

served at autopsy: unclotted blood in thoracic cavity, epididymal bleeding, nasal bleeding and subcutaneous hemorrhages; b) prolonged whole plasma prothrombin time as determined by the method of Quick *et al.*(4). Two experiments were conducted; in the first, Vit. K_1 was given at 0, 0.2, 0.4, 0.6, 0.8 and 1.0 μg/g diet. Since all levels of Vit. K_1 used in this experiment were found to be adequate, a second experiment was conductd, using the levels of 0, 0.05, 0.10, 0.15 and 0.20 μg Vit. K_1/g diet.

Results. The results are given in Table II. Coprophagy-prevented rats consuming a diet deficient in Vit. K developed severe Vit. K deficiency. This observation confirms previous reports(1,2). The results in Table II indicate that the dietary Vit. K requirement of the non-coprophagic rat was between 0.1 and 0.15 μg Vit. K_1/g of diet. When the reciprocal of the prothrombin time was plotted against Vit. K level (Fig. 1), the 3 values below the requirement fitted in a straight rising line which intersected with the horizontal line of normal prothrombin at a point corresponding to approximately 0.1 μg K/g of diet. The diet provided a growth rate of approximately 5.7 g per rat per day over the 28-day period when Vit. K was supplied, while in the case of the deficient rats (Exp. I, Table II) feed intake and growth rate were depressed.

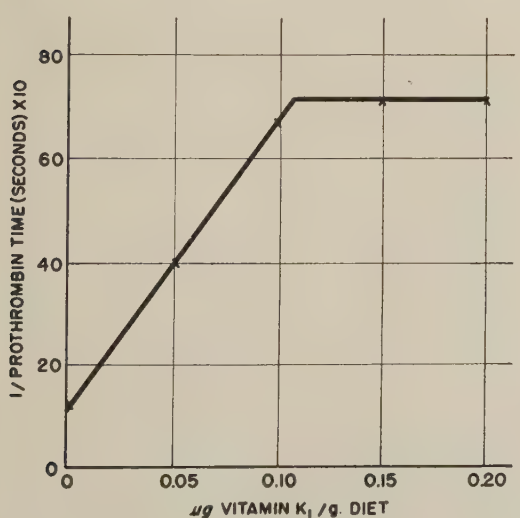

FIG. 1. 1/prothrombin time (sec.) × 10 at different levels of Vit. K_1 in the diet.

The linear relationship between reciprocal of the prothrombin time and Vit. K intake in coprophagy-prevented rats indicates that the amount of the vitamin contributed by intestinal microbial synthesis and direct absorption is insignificant. This confirms the earlier observation of Mameesh and Johnson(2) that coprophagy was the means by which Vit. K synthesized by the intestinal microflora becomes available to the rat. Growth and food intake data in Table II indicate that Vit. K was required for growth in coprophagy-prevented rats. The requirement for growth was found to be the same as that for maintainance of normal plasma prothrombin levels.

Summary. The dietary Vit. K requirement of the coprophagy-prevented growing male rat was found to be 0.1 μg/g of diet fed as Vit. K_1. This requirement satisfied the needs for maintainance of normal plasma prothrombin levels and for growth.

This study supported by contract with office of

Surgeon General, Dept. of Army. Vitamins were generously donated by Merck Sharp and Dohme, Rahway, N. J., and DL-methionine by Dow Chemical Co., Midland, Mich.

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Received December 4, 1959. P.S.E.B.M., 1960, v103.

Synergistic Action of Pneumococci and Leukocytes in Lowering Cerebrospinal Fluid Glucose.* (25527)

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The mechanism responsible for disappearance of cerebrospinal fluid sugar in bacterial meningitis has not been clearly elucidated and the relative importance of cells, bacteria, changes in meningeal permeability and accelerated utilization of glucose by neural tissue in genesis of hypoglycorrhachia remains poorly defined. It had been demonstrated that glycolysis occurs *in vitro* when CSF from dogs with non-bacterial meningitis is incubated at 37°C; the decrease in CSF sugar is proportional to number of leukocytes present (1,2). In contrast, pneumococci incubated in sterile CSF consume glucose at a very slow rate and large numbers of rapidly growing bacteria must be present before a fall in CSF sugar becomes evident. In the intact animal, however, sterile meningitis is not usually associated with a decrease in CSF glucose while bacterial infections in the meninges are almost invariably accompanied by hypoglycorrhachia. These paradoxical observations have led to the suggestion that neither cells nor bacteria can be held entirely accountable for the fall in CSF glucose(2). In our studies, CSF containing leukocytes was inoculated with pneumococci, and the mixture incubated at 37°C. Under these conditions consumption of glucose was greater than when organisms were incubated in cell-free CSF, or when CSF containing leukocytes obtained from dogs with non-bacterial meningitis was tested in absence of bacteria.

Methods. Aseptic meningitis was produced in mongrel dogs by injection of 6 ml of sterile, pyrogen-free, 0.85% NaCl into the cisterna magna. Four hours later CSF, which regularly contained 3000 to 8000 WBC/mm^3 (95-99% polymorphonuclear leukocytes), was removed by cisternal puncture. Fluids from several animals were pooled for each experiment and used immediately. Seed cultures of Type III pneumococci were stored at −70°C. For each experiment a fresh tube was thawed, added to 9 ml of tryptose phosphate broth and incubated at 37°C for 8 hours. Control tubes containing serum and broth but no bacteria were handled in similar fashion. In each experiment 0.1 ml of bacterial inoculum was added to a series of tubes containing 0.9 ml CSF obtained from animals with non-bacterial meningitis. The same number of bacteria added to 0.9 ml of sterile, cell-free CSF and fluid from animals with aseptic meningitis mixed with serum-broth inoculum not containing organisms, served as controls. One tube was immediately removed from each series for baseline bacterial and leukocyte counts and determination of glucose. The remainder were shaken in constant temperature bath at 37°C and were removed at intervals of 30, 60, 120, 180, and 240 minutes after beginning of experiment. The number of bacteria in each tube was determined by colony counts of serial 10-fold dilutions, a single colony being counted as one viable unit. Leu-

* Supported by Army Chemical Corps, Fort Detrick, Md. and by Parke-Davis Co. and Upjohn Co.

† Work performed during tenure of Lederle Medical Faculty Award.

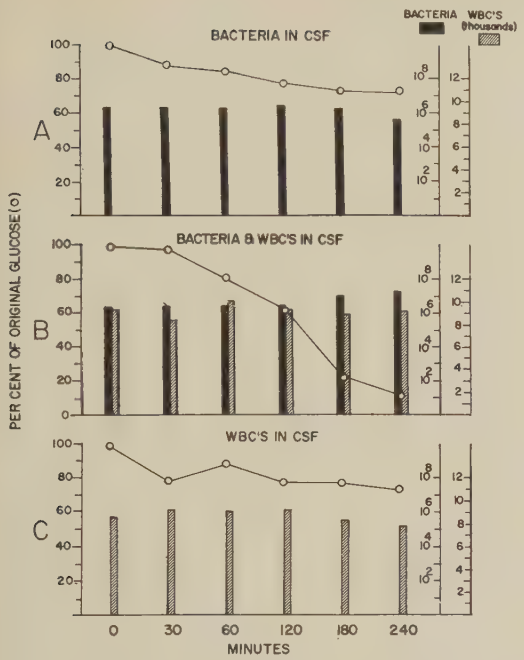
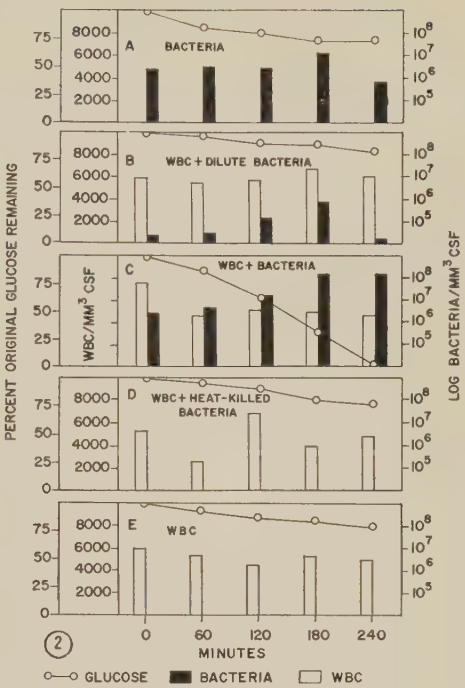


FIG. 1. Effect of pneumococci in sterile CSF, pneumococci in CSF containing leukocytes, and leukocytes in sterile CSF on CSF sugar *in vitro*.

kocytes were counted in standard manner and glucose was determined on cell free supernates by the glucose-oxidase method(3).

Results. Incubation of 10^6 pneumococci in sterile cell-free CSF resulted in very slow decrease in glucose (Fig. 1A). Similarly in CSF containing approximately 7000 polymorphonuclear leukocytes/mm^3 CSF, in absence of bacteria, glycolysis was very slow (Fig. 1C). In contrast, addition of 10^6 pneumococci to CSF containing 7000 WBC/mm^3 was followed by marked decrease in glucose which began one hour after onset of incubation; 3 hours after beginning of experiment glucose had almost entirely disappeared from the CSF. The number of leukocytes and bacteria remained relatively constant throughout but some multiplication of bacteria occurred during the last 2 hours.

The results were reproducible regularly provided a sufficient number of bacteria are present. For example, a marked decrease in glucose occurred when 5 x 10^6 bacteria were incubated with 5000 WBC/mm^3 (Fig. 2C). However, when the inoculum was diluted to contain only 5 x 10^4 organisms, glycolysis did



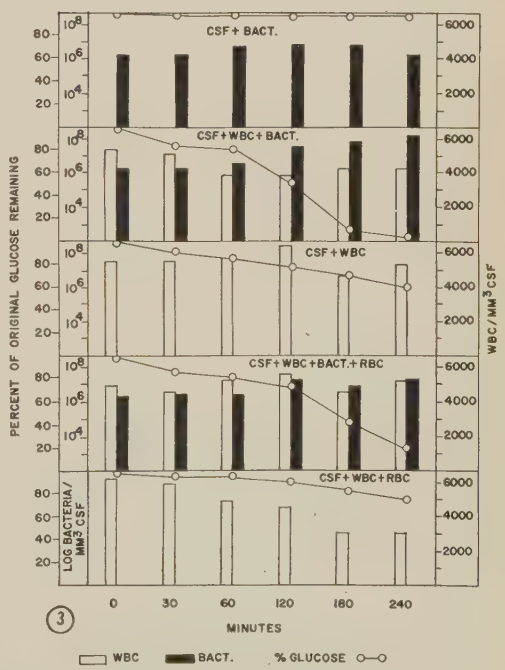


FIG. 2. Failure of dilute inoculum of pneumococci and heat-killed pneumococci to depress sugar in CSF from dogs with aseptic meningitis.

FIG. 3. Hypoglycorrhachia *in vitro* produced by combination of pneumococci and leukocytes. Erythrocytes in CSF did not influence the reaction.

not occur (Fig. 2B). In another experiment in which only 1200 leukocytes/mm^3 of CSF were present the sugar also failed to decrease despite addition of 10^7 pneumococci. This observation indicates that a sufficient number of leukocytes as well as bacteria was necessary for production of hypoglycorrhachia. These *in vitro* studies also illustrate that viable pneumococci were necessary to produce a fall in CSF sugar; addition of heat-killed bacteria to CSF containing leukocytes was not associated with a fall in glucose (Fig. 2D).

The contribution of erythrocytes or serum to the decrease in CSF glucose was evaluated in an experiment in which 2 pools of CSF each containing 5000 WBC/mm^3 were employed. In one of these 2 samples, 30,000 RBC/mm^3 were also present. The organism was grown in the absence of serum prior to inoculation into CSF. The results (Fig. 3) indicate that degree of glycolysis was not affected by presence of red blood cells.

Discussion. These studies indicate that a fall in CSF glucose can be produced *in vitro* by a combination of leukocytes and pneumococci when neither alone is capable of producing glycolysis. They also support previous observations which demonstrated that leukopenic dogs, which did not respond to pneumococcal meningitis with pleocytosis, did not manifest a decrease in CSF glucose, while normal controls showed a marked fall in sugar(4). Whether synergism between cells and bacteria occurs *in vivo* is being investigated.

The reason for the fall in glucose is not readily apparent. In some instances, at least, multiplication of organisms occurred during third and fourth hours of incubation, suggesting that the cells enhanced the nutritive value of the medium. However, a fall in glucose was noted at least one hour prior to demonstrable multiplication of bacteria, and in some experiments the bacterial count remained constant.

An alternative explanation is that in the presence of bacteria, leukocytes became actively phagocytic. It has been shown that during phagocytosis of bacteria or inert particles, leukocytes manifest increased consumption of oxygen and glucose, as well as increased production of lactic acid(5-7). It is conceivable that actively phagocyting leukocytes rather than bacteria were responsible for hypoglycorrhachia. The failure of CSF glucose to decrease when heat-killed pneumococci were added to the mixture, mitigates against this possibility but the poor environment for phagocytosis provided by a glass surface may have sharply restricted activity of the cells. As has been demonstrated in experimental pneumococcal pneumonia, phagocytosis is much more active in the intact animal than in the test tube(8). Studies are in progress to determine whether leukocytes stimulated to phagocytosis in the subarachnoid space produce hypoglycorrhachia, particularly in absence of viable bacteria.

Summary. Cerebrospinal fluid containing leukocytes was inoculated with a known quantity of pneumococci and incubated at 37°C. Under these conditions the fall in glucose was greater than when bacteria were incubated with cell-free CSF or when leukocytes were incubated in absence of bacteria. The data indicate that cells and bacteria act synergistically in producing the decrease in CSF sugar in bacterial meningitis.

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Received December 7, 1959. P.S.E.B.M., 1960, v103.

Myotrophic and Androgenic Activities of Androstanazole. A New Heterocyclic Steroid. (25528)

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Recent reports(1,2) indicated that 17β-hydroxy-17α-methylandrostano [3,2-c] pyrazole (hereafter referred to as androstanazole) is a powerful, orally active, anabolic agent. Multiple dose level assessment(3) of nitrogen retaining activity using nitrogen equilibrated castrated male rats(4), showed that androstanazole was 30 times more active than methyltestosterone when both compounds were administered orally, but when administered subcutaneously, androstanazole was only 1/20 as active as testosterone propionate. For all practical purposes, androstanazole was as active orally as subcutaneously. In recent years, use of nitrogen-balance studies with laboratory animals to assess anabolic properties of steroids has been generally abandoned in favor of simple and less tedious myotrophic assay(5,6). This report presents our observations on myotrophic and androgenic activities of androstanazole following subcutaneous and oral routes of administration. Testosterone propionate and methyltestosterone were tested concurrently with androstanazole for comparative purposes.

Materials and methods. Test materials were administered as solutions or suspensions in 10% v/v ethanol cottonseed oil, the physical state depending upon concentration and solubility. Steroids were triturated in mortar and pestle with gradual addition of the vehicle so that suspensions were finely dispersed. Myotrophic and androgenic activities were determined by modification of method reported by Hershberger, Shipley and Meyer(5). Male rats of Sprague-Dawley strain, 22 days of age (41-45 g) were castrated and maintained on lab. chow and tap water *ad lib.* in air conditioned quarters. Androstanazole and testosterone propionate were administered subcutaneously daily except Sunday, from 7th to 16th days after castration. Daily dose of each drug was contained in 0.2 ml of vehicle. Animals were autopsied on the 17th post-castration day, 24 hours after last medication. Levator ani muscle and ventral prostate were removed, blotted and weighed.

Oral studies were conducted in the same manner except that total daily dose of androstanazole or methyltestosterone was divided into 2 equal portions administered by stomach tube at 9:00 a.m. and at 4:00 p.m. each day of medication.

Results. Androstanazole was 1/7 as myotrophic and 1/33 as androgenic as testosterone propionate when both compounds were administered subcutaneously (Table I). Androstanazole was twice as myotrophic and ⅓ as androgenic as methyltestosterone when both compounds were administered orally (Table I).

Discussion. The experimental data clearly indicate that androstanazole is relatively more anabolic than androgenic. Nitrogen retention(3) and myotrophic assays indicate that androstanazole administered orally is 30 times more active in promoting nitrogen retention, 2 times more myotrophic than methyltestosterone, and is ⅓ as androgenic. Compared with testosterone propionate, subcutaneously, androstanazole is 1/20 as active in promoting nitrogen retention, is 1/7 as myotrophic and is 1/33 to 1/40 as androgenic. The interesting feature of androstanazole is the high oral anabolic activity coupled with low order androgenicity.

Androstanazole is 30 times more anabolic orally than methyltestosterone based on multiple dose level assessment of nitrogen retention in rats(3). This relative potency figure is considerably higher than the 2:1 ratio obtained from the above described results of the myotrophic assays. This difference is apparent by the larger dose of androstanazole required orally (10-40 mg/kg) to produce substantial increase in weight of levator ani

TABLE I. Relative Myotrophic and Androgenic Activities of Androstanazole, Testosterone Propionate and Methyltestosterone.

Compound	No. of animals	Dose, mg/kg/day × 9	Myotrophic response, levator ani wt, mg ± S.E.	Androgenic response, ventral prostate wt, mg ± S.E.
None	66		31.6 ± .4	6.1 ± .3
Androstanazole (s.c.)	12	1.4	35.3 ± 1.5	7.3 ± .4
	12	2.8	43.7 ± 2.5	13.0 ± .7
	12	5.6	47.9 ± 2.2	14.5 ± 1.0
	12	11.2	72.9 ± 4.0	39.6 ± 2.8
Testosterone propionate (s.c.)	12	.175	34.9 ± 1.5	19.8 ± 1.8
	12	.35	40.8 ± 2.6	33.6 ± 3.3
	12	.70	49.5 ± 1.9	57.5 ± 2.1
	12	1.40	66.8 ± 3.0	78.4 ± 6.1
Relative activity of androstanazole to testosterone propionate*			1/7	1/33
None	52		29.5 ± .9	5.1 ± .2
Androstanazole (i.g.)	6	5.25	28.6 ± 3.1	4.3 ± .7
	6	10.50	39.3 ± 4.1	8.6 ± .9
	18	21.	51.3 ± 2.4	13.4 ± .9
	12	42.	67.1 ± 3.3	24.2 ± 2.4
Methyltestosterone (i.g.)	12	5.25	31.6 ± 1.7	10.6 ± .8
	12	10.50	33.0 ± 1.5	21.9 ± 1.2
	12	21	41.9 ± 1.0	32.6 ± 1.7
	12	42	48.5 ± 2.5	45.3 ± 3.2
	6	84	63.8 ± 2.2	57.7 ± 3.9
Relative activity of androstanazole to methyltestosterone*			2	1/3

* Calculated according to method of Gaddum(7).

muscle than needed (0.5 to 5.0 mg/kg) to achieve marked improvement in nitrogen retention(3). On the other hand, the dose of methyltestosterone required for significant myotrophic and nitrogen retention (3,8) responses is identical for all practical purposes (20-80 mg/kg in each case). Early clinical observations(9) corroborate results of nitrogen retention studies in rats and imply that levator ani response does not fully reflect the anabolic potential of this new heterocyclic steroid.

Summary. Myotrophic and androgenic activities of androstanazole have been evaluated using immature castrated male rats. The data indicate that androstanazole is 1/7 as myotrophic and 1/33 as androgenic as testosterone propionate when both agents are administered subcutaneously, whereas it is 2 times more myotrophic than, and ⅓ as androgenic as, methyltestosterone when the 2 agents are given orally. The data show that androstanazole is relatively more anabolic than androgenic regardless of route of administration. The results are discussed in light of nitrogen retention data previously reported for this compound.

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Received December 7, 1959. P.S.E.B.M., 1960, v103.

Effect of Cholic and Hyodeoxycholic Acids on Metabolism of Exogenous Cholesterol in Mice.* (25529)

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Among several bile acids investigated(1), cholic and hyodeoxycholic acids had opposing effects on liver cholesterol metabolism in mice. Cholic acid increased liver cholesterol levels and decreased cholesterol synthesis; hyodeoxycholic acid decreased liver cholesterol levels, and greatly increased the synthesis rate. Investigations have shown that the influence of cholic acid on accumulation of cholesterol of exogenous origin is due to 2 factors: increased absorption and decreased cholesterol mobilization(2,3,4). It seemed possible that hyodeoxycholic acid might be of value in preventing accumulation of exogenous cholesterol, since its effects on cholesterol synthesis and levels appear to be opposite to those of cholic acid. We have therefore tested the effect of hyodeoxycholic acid on mouse liver cholesterol levels under various dietary conditions.

Methods. Eighty Webster strain female albino mice, weighing 20-25 g, were placed on a cholesterol free diet (CFD), having the following composition: vitamin-free casein, 25%; sucrose, 55%; non-nutritive bulk (alphacel†), 16%; and Phillips Hart salt mixture,† 4%. This was supplemented with following quantities of vitamins, expressed as mg/10 kg: thiamin hydrochloride, 40.4; riboflavin, 60.8; calcium pantothenate, 151.0; niacin, 1,008; pyridoxine hydrochloride, 40.4; 2-methyl-1,4-naphthoquinone, 5.12; alpha tocopherol, 30.2; folic acid, 20.3; biotin, 2.02; choline chloride, 20,200; p-aminobenzoic acid, 202; inositol, 10,100; Vit. A, 1.25 x 10^6 units. After one-week observation, mice were divided into 8 groups and placed on these diets *ad lib.*:

Group	Diet
A	CFD
B	CFD + cholic acid (CA), .5%
C	CFD + hyodeoxycholic acid (HDCA), .5%
D	CFD + cholesterol, 1%
E	*Idem* + CA, .5%
F	" + HDCA, .5%
G	" + CA, .5% + HDCA, .5%
H	" + CA, .5% + HDCA, .25%

At end of 4-week experimental period, all mice were sacrificed, livers removed and analyzed for total cholesterol(5). Stomachs and intestines were removed and cleared of all ingested material. The combined carcass, blood, stomach, and intestine were hydrolyzed by refluxing for 3 hours in 5% alcoholic potassium hydroxide. Aliquots of the hydrolysate were used for total cholesterol determination (5). Blood cholesterol levels were determined according to Sperry and Webb(6). Expired CO_2 was collected by means of Ascarite traps and determined gravimetrically.

Results. Table I presents results of this experiment. As expected, supplementing CFD with 0.5% cholic acid (Group B) caused a 51% increase in liver total cholesterol, whereas supplementation with hyodeoxycholic acid (Group C) caused a 58% decrease. Blood total cholesterol levels were 25% lower in the hyodeoxycholic acid treated animals (Group C, 74.1 $\pm$ 9.0 mg %) than in the controls (Group A, 98.3 $\pm$ 12.9 mg %). Supplementing CFD with 1.0% cholesterol (Group D) failed to increase either liver or carcass total cholesterol.

However, when 0.5% cholic acid (Group E) as well as cholesterol was added to the diet, there were significant increases in both liver and carcass total cholesterol (240% and 30%). On the other hand, addition of hyodeoxycholic acid (Group F) to the diet containing cholesterol, brought about a reduction in total liver cholesterol but had no effect on total carcass cholesterol.

A comparison of results for Group E with those for Groups G and H, indicates that adding hyodeoxycholic acid to the combination of 1% cholesterol and 0.5% cholic acid pre-

* This investigation supported in part by research grant from Michigan Heart Assn.

† Nutritional Biochemicals Corp., Cleveland, O.

TABLE I. Effect of Cholesterol and Bile Acids on Liver and Carcass Cholesterol Levels.

	Group	Total cholesterol (mg/g) Liver	Carcass*	Avg wt (g) Liver	Body
A	Control	7.30 ± .80	2.37 ±.44	2.35 ±.39	27.5 ± 2.2
B	Cholic acid (CA), .5%	11.0 ±2.48		2.08 ±.26	25.1 ± 2.2
C	Hyodeoxycholic acid (HDCA), .5%	3.05 ± .60	2.18 ±.25	2.23 ±.30	28.7 ± 1.3
D	Cholesterol, 1%	7.33 ± .97	2.74 ±.63	2.47 ±.31	27.9 ± 2.1
E	*Idem* + CA, .5%	24.7 ±6.30	3.08 ±.43	2.34 ±.37	25.8 ± 3.6
F	" + HDCA, .5%	3.33 ± .66	2.34 ±.25	2.17 ±.21	27.1 ± 1.4
G	" + CA, .5% + HDCA, .5%	6.21 ±2.10	2.36 ±.38	2.07 ±.33	24.8 ± 2.5
H	" + CA, .5% + HDCA, .25%	8.28 ±1.03	2.82 ±.27	1.98 ±.47	24.2 ± 4.1

Statistical comparison of values:

Groups	Liver	Carcass	Liver wt	Body wt
A and B	$P < .01$		$P = .08$	$P = .03$
A " C	"	$P = .28$		
A " D	$P = 1.00$	$P = .16$		
A " E	$P < .01$	$P < .01$	$P = 1.00$	$P = .27$
A " F	"	$P = 1.00$		
A " G	$P = .18$	$P = 1.00$	$P < .01$	$P = .05$
A " H	$P = .05$	$P = .01$	$P = .07$	"
E " G	$P < .01$	$P < .01$		
E " H	"	$P = .12$		
G " H	$P = .05$	$P = .01$		

* Without liver.

vents the usual accumulation of liver and carcass cholesterol. At 0.5% level (Group G), hyodeoxycholic acid prevented the accumulation of 45 mg of cholesterol per mouse liver, and 19 mg of cholesterol per carcass. While the effect at the 0.25% level (Group H) was smaller, it was still significant.

It should be noted that a diet supplemented with cholic acid caused significant decreases in liver and body weight, whereas hyodeoxycholic acid did not affect either liver or body weight.

Control of accumulation of cholesterol from both exogenous and endogenous sources is a subject of considerable interest. It has been shown(2,4) that cholesterol accumulation caused by cholic acid and its conjugates is due not only to an increase in cholesterol absorption but also to a decrease in rate of cholesterol mobilization. The present results show that in mice hyodeoxycholic acid prevents the build-up of tissue cholesterol of exogenous as well as of endogenous origin.

There are a number of possible explanations for this action of hyodeoxycholic acid: It may (a) prevent absorption of cholesterol from the intestinal lumen; (b) increase rate of hepatic cholesterol degradation and mobilization; (c) cause redistribution of liver cholesterol among other tissues; or (d) increase basal metabolic rate by affecting the thyroid gland.

Mechanism *c* does not seem possible because there was a decrease rather than an increase in carcass cholesterol at the same time that hyodeoxycholic acid prevented liver cholesterol accumulation. Mechanism *d* is eliminated because the metabolic rates of control (Group A) and hyodeoxycholic acid treated (Group C) animals did not differ significantly. CO_2 production in the 2 groups was: Group A, 1.52 ± 0.10 mg/hr/cm^2; and Group C, 1.63 ± 0.23 mg/hr/cm^2. The present experiment does not provide the necessary data to decide between mechanisms *a* and *b*.

Summary. In the female mouse fed a cholesterol-free diet: (1) supplement of 1% cholesterol did not alter liver or carcass cholesterol levels; (2) 1% cholesterol plus 0.5% cholic acid brought about large increases in liver and carcass cholesterol levels; (3) hyodeoxycholic acid reversed the effect of cholic acid and prevented cholesterol accumulation.

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Received December 11, 1959. P.S.E.B.M., 1960, v103.

Effect of Ethanol on Cytological Changes Induced by Salt Load in Nucleus Supraopticus of Rat.* (25530)

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It is generally agreed that posterior pituitary hormones are formed in the hypothalamus and transported *via* supraoptico-hypophyseal tract to the posterior pituitary lobe bound to a carrier, neurosecretory material (1). Although this hypothesis of neurosecretion is widely accepted no agreement has been reached as to site of formation of the peptide hormones or to the mechanism of their release. According to Olivecrona(2), formation of oxytocin is primarily located in the paraventricular nucleus and that of antidiuretic hormone (ADH) in the supraoptic nucleus. Conspicuous changes in ganglion cells of the supraoptic nucleus after procedures which increase blood tonicity were first noted by Ortmann(3), and later confirmed by other investigators(4). According to Ortmann(3) cytological changes in cells of nucleus supraopticus after osmotic stress with hypertonic saline are comparable with those found by Hydén(5) in nerve cells activated by other stimuli. It was further suggested that these changes reflect increase of cell activity and hence production of antidiuretic hormone. The diuretic action of ethanol has been explained as due to inhibition of the supraoptico-hypophyseal system(6,7). Kleeman *et al.*(8) in studies on ethanol diuresis in human subjects found that ethanol prevented or minimized the fall of urine flow and free water clearance that characteristically follows administration of hypertonic solutions of sodium chloride. In the present investigation the cytological changes of the supraoptic nucleus of rats induced by hypertonic salt solution were compared with those of rats receiving ethanol in addition to hypertonic salt solution.

Material and methods. White male rats weighing 150 to 170 g were used. The animals were divided into 4 groups of 7 rats. Twelve hours after withdrawal of food, rats in Group I received an intraperitoneal injection of 6 ml of water, Group II, 6 ml of 10% (w/v) solution of ethanol, Group III, 6 ml of 5% NaCl solution and Group IV, 6 ml of 5% NaCl solution containing 10% ethanol. Each rat was placed in metabolic cage and urine collected 24 hours. Rats used for histological studies were divided into 3 groups of 4 rats. 5 ml of 10% ethanol solution was administered by stomach tube twice a day during 5 days to the control group. The second group received an equal amount of 2.5% NaCl solution and the third group a solution containing 10% ethanol in 2.5% NaCl. On 6th day animals were killed by decapitation. The hypothalamic region was fixed for 24 hours in Bouin's fluid, treated with alcohol and embedded in paraffin. Transverse sections of 3 to 5 μ were cut through the supraoptic nucleus and stained according to Gomori's chromhematoxylin method.

Results. Table I shows total urine excre-

TABLE I. Total Urine Excreted in ml during 24 Hours in 4 Experimental Groups of Rats.

	Group I	Group II	Group III	Group IV
	Water	Ethanol	NaCl	NaCl + ethanol
	7.5	9.5	4.5	9.0
	7.0	9.0	5.0	8.0
	6.5	10.0	6.8	8.5
	4.5	7.5	3.5	7.0
	8.0	9.0	5.6	6.5
	6.0	8.0	5.5	7.5
	7.0	11.0	6.5	7.0
Mean	6.64	9.21	5.34	7.64

* Aided by research grants from Signe and Ane Gyllenbergs, Helsinki, Finland.

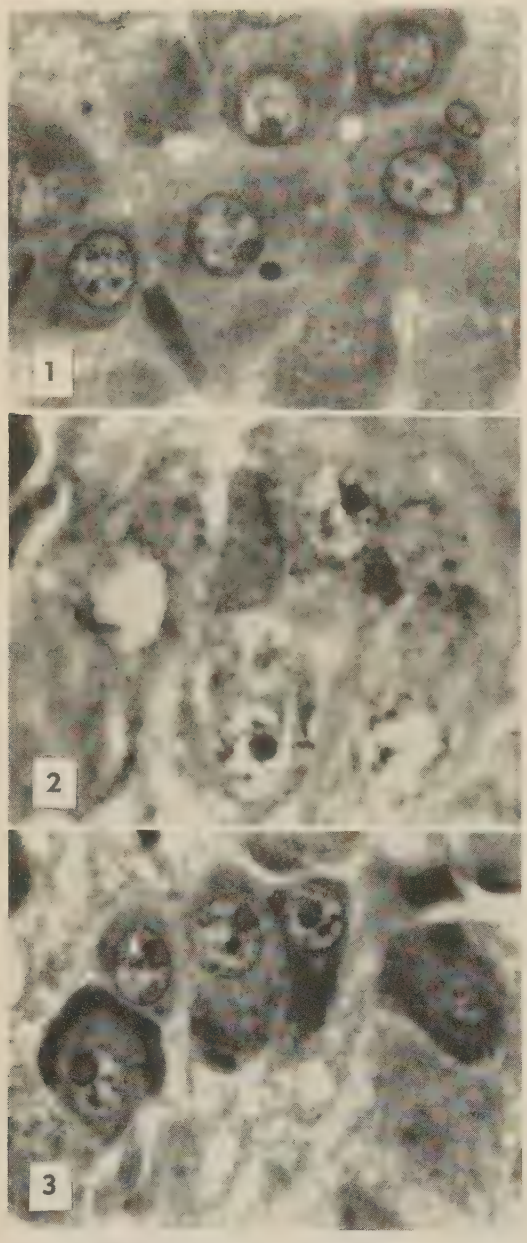

FIG. 1. Cells of supraoptic nucleus of a control rat after administration of 5 ml of a 10% ethanol solution by stomach tube twice daily during 5 days. Cells are at rest, with Nissl substance evenly distributed throughout the cytoplasm, nucleus fairly deeply stained and nucleolus small. Magnification 800 ×.

FIG. 2. Cells of supraoptic nucleus of rat after administration of 5 ml of a 2.5% NaCl solution by stomach tube twice daily for 5 days. Cells show signs of degeneration with vacuolized cytoplasm, disappearance of nuclear membrane and disintegration of nucleus. Magnification 800 ×.

FIG. 3. Cells of supraoptic nucleus of a rat after administration of 5 ml of a solution containing 10% ethanol in 2.5% NaCl by stomach tube twice daily for 5 days. Among cells in a stage of rest other cells are seen with deeply stained periphery and enlarged nucleolus. Magnification 800 ×.

tion during 24 hours in the 4 groups. When groups were statistically compared, a difference of high significance ($p<0.005$) was found between the group receiving water and the group receiving ethanol. The difference between the group receiving NaCl solution and the group receiving NaCl solution containing ethanol was also statistically highly significant ($p<0.005$).

The histological pictures of the supraoptic nucleus in the 3 groups of rats receiving ethanol, hypertonic salt solution and hypertonic salt with ethanol are seen in Fig. 1, 2, and 3. In rats of control group, ganglion cells are in a typical state of rest with the Nissl substance evenly distributed throughout the cytoplasm, the nucleus fairly deeply stained and mostly located centrally and the nucleolus small (Fig. 1).

Ganglion cells of animals receiving hypertonic salt (Fig. 2) show signs of necrobiosis and cell degeneration: vacuoles in the cytoplasm, disappearance of nuclear membrane and disintegration of the nucleus. Cells in paraventricular nuclei did not show such marked degenerative changes, and elsewhere in the central nervous system, cells appeared normal.

In rats receiving the same amount of salt as those in the previous group but in a 10% ethanol solution, degenerative changes of ganglion cells in the supraoptic nuclei were not observed (Fig. 3). Among cells in a stage of rest, other cells can be seen with a deeply stained band at the periphery of cytoplasm and with an usually enlarged and intensely stained nucleolus. According to Hydén's classification these cells appear to be in a moderate state of excitation.

Discussion. It has been shown by Eggelton (9) and Strauss *et al.*(10) that following administration of ethanol, renal tubules are capable of responding to exogenous ADH, and concluded that ethanol acts directly on the supraoptico-hypophyseal system and not upon the kidneys. Thus ethanol might affect the

osmoreceptors(11), ganglion cells of supraoptic nucleus or mechanism for ADH release. Since ethanol is distributed very rapidly over the entire water phase of the body, especially in areas of rich blood supply as the brain, and no effective osmotic pressure difference is produced between extracellular and intracellular fluid, it is unlikely that the osmoreceptors can be affected.

Whether ethanol acts directly on the ADH release mechanism, on production of ADH, or on both is difficult to conclude. Results of our study suggest that ethanol can retard development of cytological changes characteristic for exhaustion of ganglion cells in the supraoptic nuclei. Further studies are, however, needed to correlate these cytological changes with production of ADH. The action of ethanol on diuresis occurs relatively rapidly according to Kleeman *et al.*(8), this observation favoring an effect directly on the ADH releasing mechanism. According to recent theories ADH is released into blood at nerve terminals in the posterior pituitary, but whether ADH can be released into blood directly from site of its production following a stimulus is not known. Nervous impulses must also play a role in release of ADH(12) and a depression of nerve conduction with ethanol can be postulated.

Summary. Administration of ethanol to rats loaded with hypertonic salt solution increased urine flow and prevented degenerative changes in ganglion cells of the supraoptic nucleus.

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Received October 29, 1959. P.S.E.B.M., 1960, v103.

Influence of Tetrazolium Salts on Human Pseudocholinesterase.* (25531)

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Tetrazolium salts at present are used as histochemical tools primarily for measuring oxidative enzyme systems underlying cellular metabolism. Tetrazolium compounds also have a pharmacological action when administered to the intact animal. Neurotoxic effects with respiratory embarrassment, convulsions, paralysis, and death ensuing from intravenous administration of a simple monotetrazolium have been described(1). *In vivo* administration of neotetrazolium (NT) exerts a hypotensive action on renal hypertensive rats. Since blood pressure can be reduced in hypertensive states by interference with neural transmission through the sympathetic ganglia, it was postulated that NT might be acting as a ganglionic blocking agent(2). The observation that subcutaneous injection of NT results in marked deposition of formazan in the satellite and other cells of the sympathetic ganglia (3,4) lends credence to this view. It is also of interest that subcutaneous administration of various tetrazolium salts results in varying patterns of formazan deposition(5), and that INT, which is not reduced in the ganglia of the rat does not have an antihypertensive effect (unpublished data). In an effort to delineate the biochemical mechanisms underlying the neurological effects of tetrazolium

* Aided by grant from Nat. Inst. Health, USPHS.

compounds, determinations were made of the effects of 6 tetrazolium salts on human pseudocholinesterase (ChE). Since the acetylcholine-cholinesterases system is known to play a major role in transmission of nerve impulses, alteration of involved enzyme systems could conceivably account for the neurotoxic effects of tetrazolium. Koelle(6) has offered histochemical evidence for the presence of the pseudoesterase in cytoplasm of the satellite cells of the dorsal root, trigeminal, superior cervical, and stellate ganglia of the cat. In addition, pseudoesterase is more sensitive than true cholinesterase (AChE) to the inhibiting effects of various agents known to depress cellular activity(7) and is more readily influenced by a variety of tranquilizers and hallucinogens which are presumed to act within the central nervous system(8). The presence of a minimum of 3 tertiary amine groups in every tetrazolium salt also suggested a possible effect upon ChE, because this enzyme is specifically inhibited by tertiary amine groups (9).

Materials and methods. The 6 compounds tested for anticholinesterase activity were triphenyltetrazolium chloride (TTC), 2-p-iodo-3 - p - nitro-5-phenyl tetrazolium chloride (INT), neotetrazolium (NT), tetrazolium blue (BT), nitro-neotetrazolium (NNT), and the nitro derivative of blue tetrazolium (NBT). The first 2 are monotetrazoliums, the last 4 ditetrazoliums. ChE activity was determined manometrically(10) with human serum as a source of ChE. Tetrazolium salts (0.5 ml) and human serum diluted 1-50 (0.5 ml) were pipetted directly into the main compartment of the flasks, while ACh (1.0 ml) was added to the sidearm to give a final substrate concentration of 0.02 M. All flasks were adjusted to a final fluid volume of 2 ml with buffer and then flushed with 95% N_2 - 5% CO_2 for 10 minutes. The initial pH was about 7.6, and this value did not appear to change more than several tenths in either direction during the course of the reaction. Substrate was tipped into the main compartment and the flasks equilibrated for 10 minutes at a bath temperature of 30°C. Readings were taken every 10 minutes. Calculations of the velocity constants of cholinesterase activities were based on microliters of CO_2 liberated during the 1st hour. Several experiments were also carried out with benzoylcholine (0.002M) as substrate. Corrections were made for spontaneous hydrolysis. A minimum of 6 experiments was carried out with each concentration of tetrazolium salt tested.

TABLE I. Influence of Tetrazolium Salts on Cholinesterase.

Tetrazolium compound	No. of tertiary amines	Concentration	% inhibition
TTC	3	4.2×10^{-5}M	50 ± 8.2*
INT	3	2.0×10^{-5}M	48 ± 9.2
NT	6	4.2×10^{-6}M	47 ± 9.6
BT	6	1.0×10^{-7}M	42 ±10.3
NBT	6	5.0×10^{-7}M	52 ± 5.3
NNT	6	1.3×10^{-6}M	54 ± 3.6

$$^{*}\text{S.D.} = \sqrt{\frac{\Sigma d^2}{n-1}}.$$

Results. All of the tetrazolium compounds studied inhibited ChE, with BT exerting maximum effects and TTC and INT exhibiting the least inhibition (Table I). TTC and INT produced 50% inhibition at levels of the order of 10^{-5}M. Both NT and NNT produced 50% inhibition at levels of approximately 10^{-6}M, while the corresponding 50% inhibition levels for BT and NBT were of the order of 10^{-7}M. A marked decrease in magnitude of inhibition was encountered with decreasing concentrations of tetrazolium. There was a variable potentiation of ChE activity with 10^{-8}M NT which averaged 25%. Potentiation was not observed with similar low levels of the other tetrazolium salts. Addition of excess glutathione in several instances did not reverse the inhibitory effects of tetrazolium. In selected instances excess substrate (ACh) was found to reverse the inhibitory effects.

Discussion. As might be expected, the ditetrazoliums, possessing greater numbers of tertiary amine groups, exert stronger inhibitory effects than the monotetrazoliums, although each of the tetrazolium compounds tested demonstrated appreciable anticholinesterase activity. The reversal of inhibitory effects by excess substrate indicates that inhibitions are of a competitive nature. Tetrazolium compounds can effect oxidation of sulfhydryl

groups at higher pH ranges. However, since reversal of inhibition did not occur with added glutathione, the inhibition is probably not due to oxidation of sulfhydryl groups.

It has been reported that BT, which exerted maximal inhibitory effects in the present investigations, was most toxic of a series of tetrazolium salts administered to mice *in vivo* (11); this tends to support the thesis that interference with neural enzyme mechanisms underlies the neurotoxic effects. It is not likely that the toxic effects of the tetrazolium salts are due to the intracellular blocking effects of deposited formazan *per se* since this formazan remains in the ganglia long after blood pressure returns to preinjection levels in hypertensive animals treated with tetrazolium(2).

The histochemical advantages obtained by addition of the nitro groups to the tetrazolium nucleus, particularly in terms of greater sensitivity to reducing substances within tissues, are not reflected in changes in anticholinesterase activity of either monotetrazolium or ditetrazolium salts. It would appear that the redox properties of these compounds are totally unrelated to the inhibition effects observed here. Also, while some reports indicate that striking qualitative differences in activities of certain cholinesterase inhibitors exist with different choline ester substrates(12), no indication of this phenomenon was found with tetrazolium salts in the present study. Degree of inhibition and order of inhibitory effectiveness among 4 of the tetrazolium salts studied (BT, NT, INT, TTC) was essentially the same with substitution of benzoylcholine for ACh in several experiments. The exact physiological role of ChE has not been established, but the demonstrated sensitivity of this enzyme to psychotomimetic substances(13, 14) and correlation of neurotoxic effects and anticholinesterase activity in the case of the tetrazolium salts strongly suggest a fundamental significance within the central nervous system.

Summary. Neurotoxic effects of tetrazolium salts may be due to interference with enzyme systems in nerve tissue. Six tetrazolium salts were found to inhibit pseudocholinesterase, with the ditetrazoliums generally exhibiting more marked inhibitory activity than the monotetrazoliums. This may be related to the greater number of tertiary amine groups in the ditetrazoliums.

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Received November 30, 1959. P.S.E.B.M., 1960, v103.

Effects of Chlorpromazine and D-Lysergic Acid Diethylamide on Sex Behavior of Male Rats.* (25532)

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The effects of psychotropic drugs on sex behavior are of interest to understanding of drug action as well as physiological mechanisms underlying sex behavior. Soulairac and Coppin-Monthillaud(1) and Zimbardo and Barry(2) found that injection of caffeine in the male rat shortened latency to first copulation and increased rate of copulation. Furthermore, injection of 1 mg/kg of chlorpromazine prolonged latency to first copulation and decreased rate and frequency of copulation(2). The present report deals with effects of chlorpromazine and LSD-25 on sex behavior of male rats.

Method. Twelve 150 day old Sprague-Dawley male rats were kept in individual cages on 12-hour light and 12-hour dark cycle and tested during dark half of cycle. Females used as stimulus partners were brought into estrus by estrogen and progesterone† (3). They were placed with an active, nonexperimental male, and only those showing full behavioral heat were used in the tests. During tests animals were observed in semicircular observation cages 20″ in diameter with glass front. At start of test the male was allowed 2 minutes in the cage before female was introduced. If the male did not copulate within 15 minutes, and if it failed to reach ejaculation within 30 minutes, the test was terminated. Animals were selected from a larger group of males on the basis of achievement of ejaculation within 30 minutes. The mating pattern of the male(4) consists of a series of copulatory mounts with brief intromission lasting approximately ⅓ second. Each copulation is separated from the next by average interval of 45 to 60 seconds. After 8 to 15 copulations, ejaculation occurs, followed by 5 minutes, during which the male engages in no sexual activity. The following measures were made on each test: a) Copulation latency: time in seconds from introduction of female to first complete copulation. b) Number of copulations preceding ejaculation: number of mating responses resulting in intromission. c) Number of incomplete copulations: responses with mounting of female and pelvic thrusting without intromission. d) Ejaculation latency: time in seconds from first copulation to ejaculation. e) Recovery period: time in seconds from ejaculation to next copulation. Trials 1 through 10 were spaced 72 hours apart, and trials 10 through 15 96 hours apart. Each subject was allowed 1 ejaculation/trial. Before receiving any drug, each of the 12 inexperienced males underwent 4 consecutive training trials to obtain consistent, stable behavior(5). In Table I the drug, dosage conditions, and number of animals injected are listed for each trial beginning with trial No. 5. Chlorpromazine HCl‡ was injected intraperitoneally in dose of 2.5 mg/kg at concentration of 6.25 mg/cc, and LSD-25 in doses of 130 and 200 γ/kg at concentration of 1 mg/cc (1 mg = 1000 γ). Placebos consisted of equivalent volume of distilled water injected as the drug on the following drug trial. Dosage of 2.5 mg/kg of chlorpromazine depresses food intake in the rat(6). Dosage of 130 γ/kg of LSD and daily injection of this dose for 3 days between trials 13 and 14 was used to determine whether the effects of LSD on sex behavior show tolerance. Freedman, Aghajanian, Ornitz, and Rosner(7) have shown that this dosage schedule produces tolerance to impairment of rope-climbing in rats due to LSD.

Results. Both chlorpromazine and LSD reduced the number of copulations preceding

* This paper is part of dissertation for M.D. Work initiated under Dr. F. A. Beach and completed in Dept. of Psychiatry under Dr. D. X. Freedman.

† The estradiol benzoate (Progynon-B) and progesterone (Prcluton) were generously supplied by Dr. Edward Henderson of Schering Corp., Bloomfield, N. J.

‡ Chlorpromazine hydrochloride was supplied through the courtesy of Smith, Kline & French and LSD-25 through courtesy of Sandos Lab.

TABLE I. Experimental Conditions and Group Behavior for Each Trial in Terms of Observed Parameters.

Trial No.	No. of subjects	Drug and dosage	Trial No.	No. of subjects	Drug and dosage
5	12	placebo	10	12	placebo
6	"	chlorpromazine, 2.5 mg/kg 30 min.	11	11	LSD, 200 γ/kg 5 min.
			12	12	placebo
7	"	placebo	13	"	LSD, 130 γ/kg 5 min.
8	"	"			daily inj. of LSD, 130 γ/kg for 3 days
9	"	chlorpromazine, 2.5 mg/kg 30 min.	14	12	LSD, 130 γ/kg 5 min.
			15	"	placebo

Median copulation latency (sec.)	Mean No. copulations preceding ejac.	Mean No. copulations/min.	Mean No. copulatory attempts	Mean ejac. latency (min.)	Mean recovery period (min.)
20	13.5	1.21	7.4	11.2	6.3
45	9.8	.80	7.1	12.2	7.3
10	13.1	1.38	6.5	9.5	5.7
15	"	1.95	6.6	6.7	6.3
"	8.7	1.55	6.3	5.6	6.6
"	14.0	1.71	7.3	8.2	5.7
10	6.2	.91	5.2	6.8	5.5
"	11.6	1.71	3.4	"	5.1
"	8.2	1.75	6.4	4.7	5.2
"	9.2	2.24	5.8	4.1	4.7
15	11.8	1.47	5.1	8.0	5.0

ejaculation. Group performance of those that copulated and ejaculated is shown in Table I for each trial. Median rather than mean latency to the first copulation is presented as a better measure of central tendency because of skewed distribution of latencies. By comparing the score of each animal on a drug trial with his score on the preceding placebo trial, it was possible to use each animal as his own control. Only scores of those subjects that achieved ejaculation within 30 minutes on both drug trial and preceding placebo trial are included in the statistical evaluation (Table II). All 12 subjects achieved ejaculation within 30 minutes on all placebo trials. In trial 11 with LSD, 5 subjects failed to copulate, and in trial 13 with LSD 2 subjects failed to copulate. The high incidence of failure to copulate on trials 11 and 13 appeared to reflect the severity of motor disturbance induced by acute doses of LSD.

Reduction in number of copulations preceding ejaculation by LSD did not follow the pattern of tolerance described for impairment of rope-climbing by LSD(7). On trial 14, after tolerance to LSD had been presumably established, subjects still showed a low number of copulations preceding ejaculation, although they did not display the severe motor incoordination characteristic of acute effects of LSD present in trials 11 and 13.

The number of copulations/minute, or rate of copulation, was significantly reduced only on the first trial with chlorpromazine, trial 6. The low rate of copulation on trial 11 and high rate of copulation on trial 14 are sug-

TABLE II. Significance of Difference between Mean No. of Copulations Preceding Ejaculation on Each Drug Trial and Preceding Placebo Trial.

Drug tested	Trials compared	Diff. between mean No. copulations preceding ejac.	N	t	p
Chlorpromazine, 2.5 mg/kg	5-6	3.7	11	2.6	.05
Idem	8-9	4.4	12	4.0	.01
LSD-25, 200 γ/kg	10-11	7.0	6	6.6	"
LSD-25, 130 γ/kg	12-13	3.6	10	5.1	.001
Idem tolerance	12-14	2.4	12	4.6	"

gestive of treatment effects but do not approach statistical significance.

Summary and conclusions. Effects of chlorpromazine and LSD-25 on sex behavior of 12 male albino rats were studied. Chlorpromazine at 2.5 mg/kg injected 30 minutes before trial and LSD at both 200 γ/kg and 130 γ/kg injected 5 minutes before trial, reduced the number of copulations preceding ejaculation. Reduction in number of copulations by LSD did not follow the pattern of tolerance described for impairment of rope-climbing by LSD. Rate of copulation on the first trial that chlorpromazine was given was significantly slowed.

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Received June 30, 1959. P.S.E.B.M., 1960, v103.

Water, Neutral Fat and Solids of Adipose Tissue.* (25533)

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It is variously stated that water accounts for from 5 to 30% of the weight of adipose tissue(1). The amount of water associated with fat is a variable which may influence values obtained for lean body mass and total body fat calculations when these parameters are estimated from densitometric or from total body water measurements(2). In this connection the question arises, does adipose tissue increase in size and weight as the result of addition of both water and neutral fat or are neutral fats incorporated into adipose tissue without inclusion of significant amounts of additional water? Since adipose tissue can make up from about 5 to 50% of an individual's total body weight, it is of interest to determine the factors which may influence the variability of adipose tissue composition. This report presents the results of an investigation in which relationships among neutral fat, solids and water in adipose tissue were studied.

Methods. Twenty-four adult male Sprague-Dawley rats were subjected to a cold environment (4-8°C) from 23 to 26 days; 12 control animals were maintained at room temperature (22-24°C). In both groups water and food (Purina Dog Chow) were given *ad lib.* Peri-renal adipose tissue was removed as completely as possible from etherized, exsanguinated, adult white male Sprague-Dawley rats. Water was estimated by weight difference after drying *in vacuo* (40-60 mm Hg subatmosphere pressure) at 38°C for 48 hours. Neutral fat was estimated after extraction with ethyl ether. The weight of the dry, defatted residue represents solids present in the adipose tissue.

Results. The weight of peri-renal adipose tissue in experimental and control animals fluctuates as a function of individual variation and/or as the result of exposure to low temperature for prolonged periods. As weight of the adipose tissue increases, the percent water decreases exponentially, whereas the percent neutral fat increases exponentially (Fig. 1). Large samples of adipose tissue contain about 5% water and 95% neutral fat whereas in smaller samples there may be 30-40% water, and only 50-60% neutral fat.

In an attempt to study these relationships further, data from both experimental and con-

* This work supported by Research Grant from Wisc. Heart Assn.

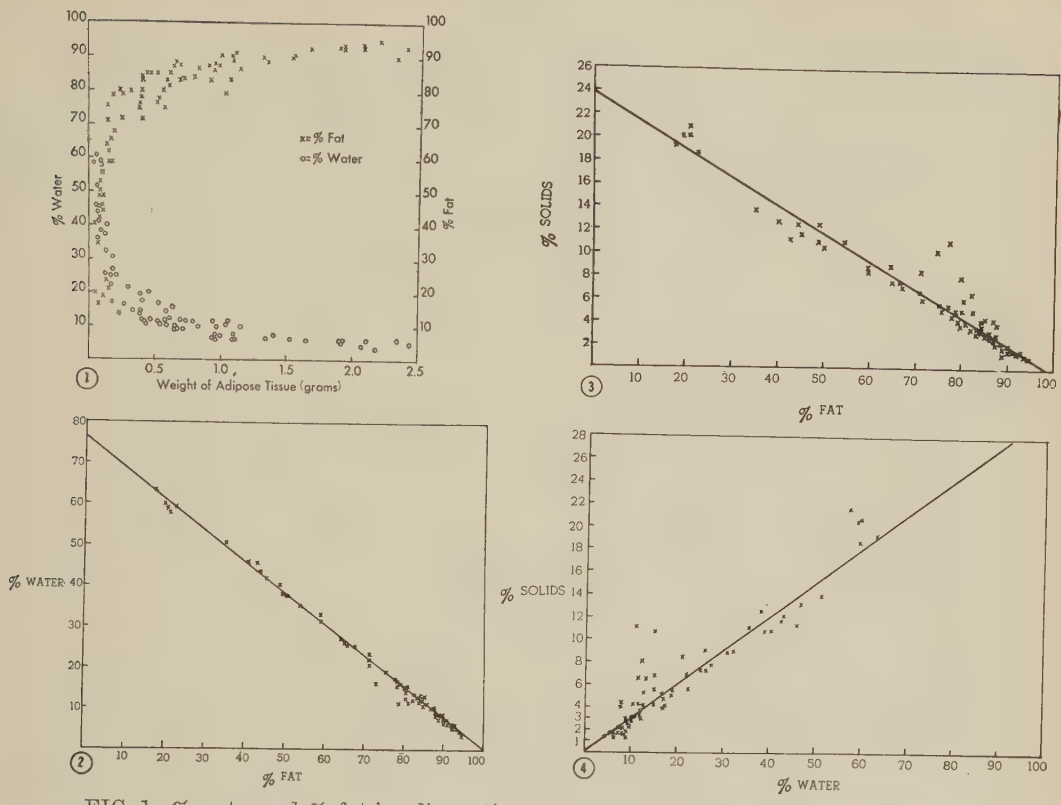

FIG. 1. % water and % fat in adipose tissue.
FIG. 2. Relationship between % fat and % water in adipose tissue. $y = -.76$, $r = -.99$.
FIG. 3. Relationship between % fat and % solids in adipose tissue. $y = -.25 + 24$, $r = -.96$.
FIG. 4. Relationship between % water and % solids in adipose tissue. $y = .3 \times + .17$, $r = .95$.

trol animals are plotted together. In peri-renal adipose tissue an inverse relationship exists between the % water and % fat (Fig. 2). If one extends the best fit line to the X axis a hypothetical situation is created in which "adipose tissue" consists of 100% neutral fat. Conversely if the best fit line is extended to the Y axis this tissue contains no neutral fat and about 76% water. An inverse relationship also exists in this tissue between % fat and % dry solids (Fig. 3). A similar extension of the best fit line to its intercepts suggests that in "adipose tissue" free of solids, there is about 98% neutral fat, whereas, in "adipose tissue" free of neutral fat there is 24% dry solids. The percentages of water and dry solids in peri-renal adipose tissue are directly related (Fig. 4).

The % water in fat free peri-renal adipose tissue is significantly lowered in the cold treated animals—mean of 80.39 ± S.D. 1.7 in controls and 77.12 ± in cold exposed animals. The Student's T test indicates this difference is significant at the 1% level.

Discussion. It may be rewarding to examine some relationships between water, solids and neutral fat in peri-renal adipose tissue. It has been demonstrated that water and dry solids vary inversely with amount of neutral fat present in adipose tissue and directly with one another. "Adipose tissue" freed of neutral fat contains a constant amount of water and dry solids; as water and dry solids approach zero neutral fat approaches 100% of the composition of adipose tissue. These observations can be taken to support the hypothesis that neutral fat is added to a tissue matrix without addition of water. Water it seems exists in this tissue in association with non-fat solids, since addition of neutral fat

acts to dilute these constituents. The above data lend further support to the hypothesis that the % water in the fat free solids of adipose tissue is constant.

The recent observation(3) that an increase in density of adipose tissue occurs as the result of loss of fat during caloric restriction in humans could be interpreted by the above data.

The decrease in % water in fat free solids of cold treated animals reflects the contraction of extracellular fluid volume as the result of cold exposure(4).

Summary. Peri-renal adipose tissue may contain varying amounts of water, neutral fats and solids. The absolute amount of these constituents is a function of weight of tissue. Cold exposure in addition to depleting adipose tissue of neutral fat also decreases water content of fat free solids. Water and solids vary directly with each other and both vary inversely with amount of neutral fat present. It appears that water present in adipose tissue is located in tissue solids and is not associated directly with neutral fats; and that when neutral fats are added to adipose tissue they are added, with limits of this experiment, independently of water.

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Received July 7, 1959. P.S.E.B.M., 1960, v103.

Effect of Dietary Protein Quality on Alteration of Serum Proteins and Lipoproteins in the Rat.* (25534)

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The physiological importance of serum proteins and lipoproteins is not fully understood. Besides providing for maintenance of osmotic pressure(1) in serum, they provide transportation mechanisms for many water insoluble nutrients such as Vit. A(2), cholesterol(3) and steroid hormones(4) to sites of tissue utilization. While many infectious diseases have been shown to alter markedly the protein fractions of sera, comparatively little attention has been given to nutritional influences. In this study protein from several single sources was fed to rats to determine effects on serum protein and lipoproteins.

Method. Four groups of rats, 4 male and 6 female, 48 days old, were fed purified rations that contained casein, lactalbumin, gluten and zein respectively as primary protein source (Table I). After 38 days the rats were sacrificed and livers and kidneys removed and immediately weighed. Serum proteins and lipoproteins were electrophoretically separated on paper strips in a Durrum cell in sodium veronal buffer (pH = 8.6; u = .076) at 2.5 ma. for 16 hours. Serum proteins were dyed with bromphenol blue while lipoproteins were dyed with oil red O for 24 hours at 37°C. The per cent of total serum and lipoprotein of each fraction was determined by scanning with an analytrol (Beckman Instrument Co.)

TABLE I. Composition of Experimental Diets.

Constituents	Ration No. 1	2	3	4
	g			
Casein	180			
Lactalbumin		192.1		
Gluten			188.9	
Zein				190.1
Mineral mix	40	40	40	40
Brewers yeast	80	80	80	80
Fiber	50	50	50	50
Sucrose	600	584.8	594.9	575.6
Fat	50.0	53.1	46.2	64.3

* Arizona Agric. Exp. Station Technical Paper No. 545.

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TABLE II. Effect of Sex and Dietary Protein Quality on Liver, Kidney, Serum Proteins and Lipoproteins of Rats.

	Dietary protein				Sex	
	Casein	Lactalbumin	Gluten	Zein	♂	♀
Wt change, g						
Initial	102.0	101.7	99.4	100.2	104.6	98.5
Final	162.3	175.5	168.6	104.3 †	177.6	135.9
Kidney wt, g	.627	.649	.684	.741†	.655	.688
Liver wt, g	3.86	3.76	3.63	3.31	3.50	3.59
Protein, %	60.2	64.1	58.0	55.8	57.4	60.9
Dry matter, %	34.5	31.2	39.2	36.2	37.2	34.6
Serum proteins, %						
Albumin	64.2	65.9	67.6	61.5 †	66.7 *	63.6
Alpha globulin	7.8	7.4	8.3	9.3	8.5 *	7.9
Beta "	16.3	13.1	15.5	16.8 †	14.8	15.8
Gamma "	11.7	13.6	8.6 †	12.4	10.0	12.7 *
Lipoproteins, total	72.6 †	50.6	60.7 †	33.8	55.3	51.7
Fraction III, %	25.3	21.2	11.2 †	6.1 †	13.8	20.1 *
II, %	63.8	73.5 †	74.9 †	55.0	72.9 *	61.4
I, %	10.9 †	5.3 †	13.9	38.9	13.3	18.5

* $P < .05$. † Significantly different from rats fed other source of protein ($P < .05$).

All data were subjected to analyses of variance and Duncan's Multiple Range Test for significance(5).

Results. Sex did not influence kidney or liver weight or liver protein and dry matter composition. Almost all serum protein and lipoprotein fractions were significantly influenced by sex (Table II).

Although serum protein fractions did not vary greatly in magnitude, zein-fed rats exhibited a significantly lower serum albumin than rats fed protein from other sources. Zein-fed rats were not significantly different in beta globulin % from rats fed casein. Beta globulin % of rats fed zein was significantly lower than in rats fed gluten. Gluten-fed rats exhibited significantly lower gamma globulin than rats fed protein from other sources.

Total lipoprotein concentration of rat sera was estimated from total analytrol counts, indicative of total dye concentration. Sera from casein-fed rats possessed the greatest lipoprotein concentration (Table I), but this concentration did not differ statistically from that of gluten-fed rats. Lipoprotein content of zein-fed rat sera was significantly lower than that of sera of rats fed protein from any other source. Characteristic lipoprotein fractions of rats fed protein from various sources are presented in Fig. 1. When based on % of total lipoprotein the slowest moving lipoprotein fraction (III) was similar for rats fed casein and lactalbumin. However, these 2 dietary proteins resulted in significantly higher fraction III than either gluten or zein.

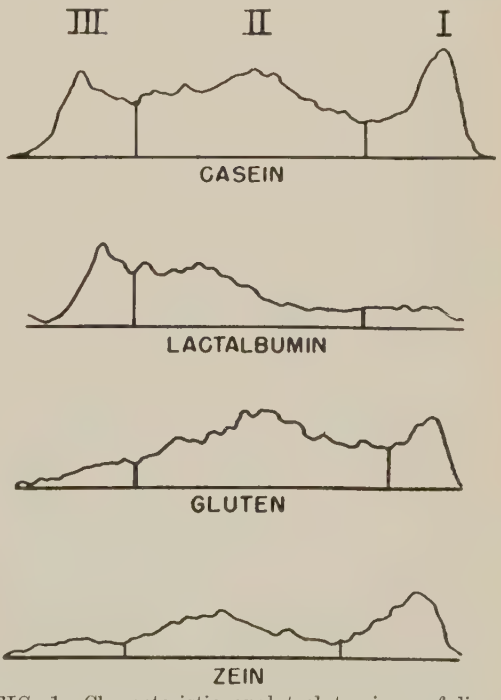

FIG. 1. Characteristic analytrol tracings of lipoprotein fractions from sera of rats fed various types of pure proteins. Fraction I was the fastest moving fraction and similar in mobility to albumin.

Sera of rats fed zein possessed significantly lower concentrations than sera from rats in any other group. Fraction II of rats fed lactalbumin and gluten was significantly higher than that of rats fed casein or zein. A significantly smaller percentage of fraction II was found in sera of zein-fed rats than in the sera of any other group.

Discussion. The importance of amount and proportion of serum proteins and lipoproteins in sera of animals is only partially understood. Diets were formulated similar to those fed by James and El Gindi(6) who reported that protein quality markedly influenced carotene utilization in the rat. Since Erwin, *et al.*(3), and others have found that albumin binds Vit. A and carotene and probably forms a transportation mechanism for these nutrients, it appeared likely that the effect of dietary protein quality was mediated through alteration in albumin content of the sera. However, the effect of dietary protein quality on alteration of albumin proportion in total serum protein could not account for differences observed in carotene utilization by the rat. Unfortunately, total serum albumin per cent was not measured.

Several investigators(7) observed that quantity of dietary protein altered serum cholesterol content. More recently, others(8) found that quality of dietary protein also influenced the level of serum cholesterol. Serum cholesterol has been found to be largely associated with certain lipoprotein fractions and, in rats, with alpha lipoprotein. The results of this study suggest that the influence of dietary protein quality on serum cholesterol is mediated through alteration of quantity and type of serum lipoproteins. That is, dietary protein affects the transportation mechanism for cholesterol and probably does not contribute to its synthesis.

Summary. Serum proteins and lipoproteins were fractionated in sera from male and female rats fed 4 pure protein sources respectively. Almost all lipo- and serum protein fractions were affected by sex. Serum protein fractions did not vary greatly in magnitude but statistically significant changes were attributed to dietary proteins. Type of dietary protein markedly altered the fractions of lipoproteins in the rats.

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Received September 21, 1959. P.S.E.B.M., 1960, v103.

Effect of Carbon Tetrachloride on Release of Free Fatty Acids by Rat Adipose Tissue.* (25535)

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Calvert and Brody(1) presented evidence that the mechanism of carbon tetrachloride hepatotoxicity involves a primary action on the central nervous system. According to their hypothesis carbon tetrachloride administra-

* Supported by grants from Nat. Heart Inst. and from Nat. Inst. of Arthritis and Metab. Dis., N.I.H., P.H.S.

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TABLE I. Effect of Subcutaneously Injected Epinephrine on Release of Free Fatty Acids from Rat Epididymal Adipose Tissue.

		No. of animals	Min.*	FFA released†	Significance‡
Exp. I	Controls	6		3.22 ± .51	
	Epinephrine, 1 mg/kg	6	30	5.54 ± .75	$P < .05$
	Idem	6	60	3.52 ± .52	$P > .50$
Exp. II	Controls	6		6.72 ± .76	
	Epinephrine, .1 mg/kg	5	30	9.74 ±1.15	$.1 > P > .05$
	" 1.0 "	7	"	10.50 ± .67	$P < .005$

* Time elapsed from epinephrine inj. to sacrifice of rats.
† Free fatty acids released, μeq/g of adipose tissue in 3 hr, mean ± stand. error.
‡ Statistical significance of data was determined by comparing mean values for experimental groups with mean values of corresponding control group, in Student's t test.

tion evokes a sympathoadrenal discharge. The released catechol amines are believed to cause liver necrosis due to hepatic vasoconstriction and a fatty liver resulting from accelerated release of lipid from adipose tissue. One means by which lipid of adipose tissue is mobilized is as free fatty acids‡ bound to plasma albumin (2). Release of these fatty acids from adipose tissue is influenced by catechol amines. Administration of epinephrine and norepinephrine to human subjects and to dogs results in an elevated level of plasma free fatty acids (3-6). Further, addition of both these amines *in vitro* to the excised epididymal fat pad stimulates release of free fatty acids (7,8). If the epididymal fat pad from epinephrine treated rats exhibited an increased rate of release of free fatty acids *in vitro* this tissue would provide a method for testing whether administration of carbon tetrachloride causes a sympathoadrenal discharge.

Methods. Male albino rats of Sprague-Dawley strain, 113-190 g, previously fasted for 16 hours, were used. The animals were selected so that in each experiment maximum weight range was no more than 35 g. Carbon tetrachloride was administered *via* stomach tube under light ether anaesthesia as a 1:1 mixture with mineral oil, at dose of 0.5 ml of mixture/100 g body weight. Two control groups were employed, except where noted. One group was untreated, except for starvation; the other received 0.25 ml of mineral oil orally under light ether anaesthesia. Epinephrine (adrenalin in oil, Parke, Davis and Co., 1:500) was given subcutaneously at dose of 1 mg or 0.1 mg/kg of body weight. Control animals received 0.1 ml of peanut oil. Animals were decapitated, epididymal fat bodies removed, weighed, and incubated for 3 hours at 36°C in Krebs-Ringer phosphate solution containing 5% albumin, at pH 7.4. Free fatty acids released were determined as previously described (9).

Results. Administration of epinephrine to rats at a dose of 1 mg/kg 30 minutes prior to removal of epididymal fat bodies elicited a significant increase in release of free fatty acids into the medium (Table I). No significant change was observed in free fatty acids released when animals were given the same dose of epinephrine, but killed 60 minutes after injection. When epinephrine was given at a dose of 0.1 mg/kg 30 minutes before sacrifice an increased release of free fatty acids was observed, but the significance statistically was low.

Release of free fatty acids from adipose tissue excised from rats at various times after carbon tetrachloride administration showed no significant changes in comparison with untreated controls or to the control group which received mineral oil alone (Table II).

These time periods were chosen for the following reasons. Increased release of free fatty acids *in vitro* is evident 30 minutes after epinephrine administration. A significant increase in chemically determined hepatic fat is not evident until 3 to 4 hours (10), and the time at which liver fat is increasing maximally is about 9 hours following carbon tetrachloride feeding.

Discussion. Dole (3) and Gordon and

‡ The term free fatty acids is intended to have the same meaning as unesterified or nonesterified fatty acids.

TABLE II. Effect of CCl_4 Poisoning on Release of Free Fatty Acids from Rat Epididymal Adipose Tissue.

Exp.	Treatment of animals	No. of animals	Hr*	FFA released†
1	Control	6		5.12 ± .53
	Mineral oil	6	.5	4.70 ± .43
	CCl_4	6	.5	5.48 ± .64
2	Mineral oil	6	4	7.72 ± .61
	CCl_4	6	4	6.01 ± .76
3	Control	7	9	5.39 ± .58
	CCl_4	8	9	5.29 ± .60

* Time elapsed from CCl_4 administration to sacrifice of rats.

† Free fatty acids released, μeq/g of adipose tissue in 3 hr, mean and stand. error. All experimental mean values were not significantly different from corresponding control values in Student's t test.

Cherkes(4) showed that administration of epinephrine to human subjects was followed by marked, prompt, and short-lived rise in plasma levels of free fatty acids. Gordon and Cherkes(7) subsequently showed that when epinephrine was added to rat epididymal adipose tissue *in vitro* there was an accelerated release of free fatty acids into the incubation medium. The data of Table I extend these observations to show that epididymal adipose tissue excised from rats given epinephrine subcutaneously exhibits an increased rate of release of free fatty acids. These data establish that the change in adipose tissue metabolism induced by epinephrine *in vivo* persists in the excised tissue *in vitro*.

With regard to the suggestion of Calvert and Brody(1) that carbon tetrachloride elicits a discharge of the sympathoadrenal system, it seems reasonable to expect that epididymal adipose tissue of rats poisoned with carbon tetrachloride should exhibit an increased rate of release of free fatty acids *in vitro*. This expectation was not realized (Table II); however, these data cannot be considered as sufficient to disprove the hypothesis. It is possible that in carbon tetrachloride poisoned rats, a small continuous discharge of epinephrine is producing a continuous accelerated release of non-esterified fatty acids from adipose tissue, and that the alteration in metabolism of the adipose tissue does not persist *in vitro*. However, that this is unlikely is shown by the observation of Spitzer and Miller(11) that plasma free fatty acids dropped sharply and remained depressed for 48 hours after subcutaneous administration of carbon tetrachloride to dogs. Thus the interesting and provocative data reported by Calvert and Brody(1) may involve mechanisms other than those suggested by these authors.

Conclusions. 1. Administration of epinephrine to rats increased release of free fatty acid from adipose tissue *in vitro*. 2. Administration of carbon tetrachloride had no effect on release of free fatty acids from adipose tissue *in vitro*. 3. These results are not consistent with the hypothesis that carbon tetrachloride administration elicits a supporting adrenal discharge.

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Received October 9, 1959. P.S.E.B.M., 1960, v103.

Conditions Affecting Inhibition of Tricarboxylic Acid Cycle by Fluoroacetate in Rat Liver Mitochondria.*† (25536)

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Earlier reports(1,2) indicated that fluoroacetate (FAc) poisoning in male rats did not lead to citrate accumulation in the liver, except when animals were castrated or exposed to X-ray irradiation(3). Various reasons(4, 5) were suggested for this lack of citrate accumulation. Experiments described here were undertaken to find out the response of rat liver mitochondria to fluoroacetate, and dependence of this response on various factors such as sex, nutritional state, X-radiation and 2:4-dinitrophenol.

Materials and methods. Wistar male rats, of 150 to 300 g weight maintained on Purina standard rat chow were used. *Starving conditions,* where used, entailed withdrawal of food 16-18 hours prior to experiment, with water furnished *ad lib.* In irradiation experiments animals received total body irradiation of 900 Roentgens at 30 r/minute. The characteristics of the machine were 250 kv, 15 milliamps with filters of 0.21 mm copper inherent, and 0.5 mm parabolic 1 mm aluminum. Irradiated animals were in groups of 6 and rotated 1 rpm. In all experiments, the animals were killed by cervical fracture and gross venesection. Livers, prior to excision, were immediately exposed and perfused *via* portal vein with about 60 ml of ice-cold 0.16 M KCl. Homogenization and/or fractionation of subcellular components adhered to methods described by Smith(6). Each Warburg flask contained 1 ml of basic medium composed of Phosphate pH 7.2 (0.06 M), $MgCl_2$ (0.006 M), and KCl (0.12 M). To this was added 1 ml of mitochondrial suspension in either 0.29 M mannitol or 0.25 M sucrose, with substrates added as indicated in specific experiments to give final volume of 3 ml. $Qo_2(N)$ values were determined by Warburg direct method at 37°C, with oxygen as the gas phase. All chemicals were of reagent grade. Fluorocitric acid (FC) was synthesized by the method of Rivett(7) and purified chromatographically using n-propanol, ammonia and water (6:3:1) (8). After air drying of chromatograms the acids were made visible by spraying(9) 2 test strips cut out 5 cm from opposite ends of streak. The fluorocitric acid preparation showed no trace of oxalic acid; however, it gave 3 distinct spots of unequal intensity. The lowest of these, Rf 0.32, corresponded to fluorocitrate as shown by comparison with enzymically formed fluorocitrate. This portion, representing 60-70% of original material, was now cut out as a strip parallel to starting line and eluted with distilled water. The eluates of several strips were pooled and lyophilized to 1 ml volume. Traces of ammonia and ammonium salt contained in this were removed by passage through a 1 x 15 cm Amberlite IR-120 column (hydrogen form). The free fluorocitric acid in the eluate was titrated with 0.1 N NaOH to pH 7.4 and lyophilized to dryness. The salt was kept in a vacuum desiccator over P_2O_5. A solution of this salt gave a single spot of Rf 0.32 to 0.35. ATP disodium salt was obtained from Pabst Labs, Milwaukee, Wisc. Testosterone and progesterone were purchased from Nutritional Biochemicals Corp., Cleveland, O. Nitrogen determinations were carried out by standard micro-Kjeldahl methods. Citrate was determined by the method employed by Brady, Mamoon and Stadtman(10), except that in our assays 0.3 ml of 5% (w/v) potassium permanganate was added dropwise and the color was developed with fresh solution of 5% (w/v) sodium sulfide.

Results. It was suggested(4) that absence of citrate accumulation in the liver of fluoro-

* This work was supported by grants from Nat. Inst. Neurol. Dis. and Blindness, N.I.H., U.S.P.H.S., and by a grant from Am. Cancer Soc. and by Cancer Research Funds of Univ. of California.

† The authors express thanks to Dr. P. P. Noyes for help with radiation experiments, and to E. Roth, T. Roark and I. Rask for skillful assistance.

TABLE I. Recovery of Citric Acid Following Decapitation or Freeze Killing of Animals.

	Citrate (μg/g wet tissue)	
	Decapitation	Liquid air freeze
Liver	31	32
Kidneys	725	810
No. animals	4	4

Animals were sacrificed 2 hr after intraper. administration of sodium fluoroacetate (5 mg/kg).

acetate poisoned rat was due to *in situ* destruction of this metabolite following sacrifice of the animal. To reexamine this latter point 2 groups of male rats were injected intraperitoneally with 5 mg/kg fluoroacetate. Two hours later one group was killed by decapitation, the liver and kidneys immediately removed and analyzed for citrate. The other group was killed by immersion of head in liquid air, and after exposing the viscera with a median incision, the whole animal was dropped in liquid air. Organs were thus immediately frozen solid. The frozen liver and kidneys were removed, powdered, and homogenized in ice-cold TCA. The results of citrate analyses (Table I) show that citrate content of livers of the 2 groups did not differ significantly. According to earlier observations (11) the liver of fluorocitrate poisoned rats does not destroy accumulated citrate to any significant degree.

Effects of starving on citrate accumulation and respiration in presence of fluoroinhibitors are shown in Table II. In starved animals there is a general increase of respiration. It is obvious that the inhibitory ratios, FAc/

TABLE II. Effect of Starving on Mitochondrial Respiration and Citrate Accumulation.

	Fed		Starved	
	Qo_2 (N)	Citrate,* μMole/N	Qo_2 (N)	Citrate,* μMole/N
Control	94	.36	187	.48
Fluoroacetate	74	1.94	143	1.66
Fluorocitrate	39	5.32	26	2.20
No. experiments	6		10	

Substrates and inhibitors in the flasks were: sodium fumarate (20 μMole), sodium pyruvate (9 μMole), ATP (.5 μMole), sodium fluoroacetate (3.3 μMole), synth. trisodium fluorocitrate (.27 μMole). 1 ml of mitochondria suspended in .29 M mannitol.

* Citrate values were calculated/mg mitochondrial nitrogen.

Control, are extremely close in both groups, namely 0.785 in fed and 0.763 in starved rats. Greater respiratory inhibition produced by fluorocitrate in the starved animal is not reflected in the citrate accumulation. The latter is in keeping with observations of others (12) who found that starvation reduced citrate accumulation in the liver of fluorocitrate poisoned rats. Investigation of conditions of inhibitory action of fluorocompounds is complicated by a time lag of 20-30 minutes before the effect on respiration of both homogenate and mitochondrial system becomes noticeable (Fig. 1). The appearance of respiratory in-

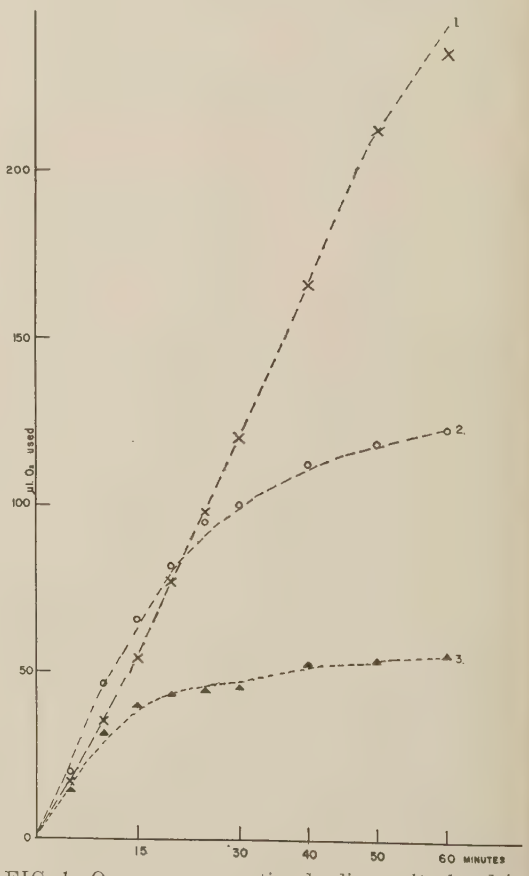

FIG. 1. Oxygen consumption by liver mitochondria obtained from starved rats. (1) Control. (2) Sodium fluoroacetate (1.1×10^{-3} M). (3) Synth. trisodium fluorocitrate (5×10^{-5} M). Substrates and conditions are as given in Table II.

hibition by 0.1 to 0.2 mM fluorocitrate will set in within the first 15 minutes.

Effect of sex and sex hormones. Reports of *in vivo* experiments do not completely agree

TABLE III. Effect of Sex and Sex Hormones on Respiration and Citrate Accumulation.

	Additions	Respiration Q_{O_2} (N) ♂	Respiration Q_{O_2} (N) ♀	Citrate, μMole/mg N ♂	Citrate, μMole/mg N ♀
a)	None	159* ±16†	217 ±20	.73 ± .13	1.19 ±.06
	FAc	163 ± 7	184 ±31	2.62 ± .50	2.36 ±.35
	T	122 ± 5.5	157 ±23	.53 ± .11	.99 ±.16
	" + FAc	104 ± 1	130 ±13	1.82 ± .41	2.73 ±.23
	P	108 ± 2	131 ±17	.55 ± .09	1.07 ±.09
	" + FAc	88 ± 2.5	91 ±10	1.19 ± .31	1.60 ±.05
b)	None	157 ± 2.5	262 ±42	1.78 ±1.09	4.31 ±.80
	FAc	159 ±25	263 ±12	2.10 ± .74	4.84 ±.56
	T	127 ± 5.5	208 ±23	1.53 ±1.04	3.46 ±.53
	" + FAc	128 ±13	227 ±22	2.16 ± .75	5.04 ±.26
	P	101 ± 5	132 ±20	.90 ± .50	1.47 ±.15
	" + FAc	100 ± 4.7	120 ± 8	1.27 ± .10	1.98 ±.11
No. animals		6	6	6	6

All flasks contained fumarate (40 μM), ATP-Na_2 (4 μM). Under b), sodium pyruvate (20 μM) was additionally given. Other additions as indicated: sodium fluoroacetate (FAc) 3.3 μM, testosterone (T) 1.6 μM, progesterone (P) 1.6 μM. Steroid solvent was prepared as described by Wade and Jones (17). Mitochondria were suspended in sucrose (.25 M).

* Mean. † Stand. error.

that there is a sex difference in response of rat liver to fluoroacetate poisoning. Our experiments were designed to study the *in vitro* existence of any such difference. Some results are given in Table III. Statistical comparison of the significance of differences both with respect to respiration and citrate accumulation between mitochondria of either sex revealed no difference with fumarate as substrate. In presence of fumarate the mitochondrial system of either sex was able to accumulate citrate; whence both sexes are able to convert fluoroacetate to fluorocitrate. However this effect is abolished by pyruvate. Pyruvate moreover leads to a significant increase of citrate accumulation in the female system only. Addition of sex hormones did not bring out any difference in response with respect to respiration or citrate accumulation. Addition of sex hormones alone did not lead to citrate accumulation. However progesterone prevented fluoroacetate-produced citrate accumulation to a significant degree in liver mitochondria of male rats, but only to a 5% level of fidelity in female rats. Progesterone alone or in combination with fluoroacetate inhibited respiration in both sexes.

Effect of irradiation. It was suggested(13) that the increased accumulation of citrate in the liver of irradiated male rats was due to effect of X-ray radiation on the metabolic pathways related to citrate oxidation which were specific to male rats. Table IV summarizes results obtained from a comparison of liver mitochondria of male rats of different nutritional states with those of rats exposed to irradiation. Statistical analysis of the results revealed that in presence of fluoroacetate citrate accumulation increased in the mitochondria of irradiated animals only in comparison with those of starved rats ($p<0.03$) but not with those of fed rats ($p = 0.05$). This agrees well with the *in vivo* findings(13).

Effects of 2:4-dinitrophenol and fluoroinhibitors. As a corollary to earlier studies on comparative effects of dinitrophenol (DNP) and the fluoroinhibitors(14,15) some values were obtained on citrate accumulation and respiration with fluoroinhibitors in presence of DNP (Table V). When DNP and fluorocompounds are simultaneously added to the system inhibition not only becomes greater with respect to respiration but to citrate accumulation as well. Judah and Rees(16) described the effect of DNP as protecting citrate oxidation and showed that it was due to failure of fluoroacetate activation through lack of ATP resynthesis. They gave no information on citrate accumulation, consequently it is uncertain whether the DNP concentration re-

TABLE IV. Effect of X-radiation on Citric Acid Levels of Rat Liver Mitochondria.

	Fed	Starved	Irradiated
FAc	340 ± 35*	241 ± 24	793 ± 213
No. animals	6	5	10

System as described in Table II.

* Mean ± stand. error.

TABLE V. Effect of 2:4-Dinitrophenol and Fluorocompounds on Mitochondrial Respiration and Citrate Accumulation.

Additions to system	Q_{O_2} (N)	Citrate, μMole/mg N
None	94	.07
DNP	120	.08
FAc	75	1.35
" + DNP	70	.92
FC	39	3.35
" + DNP	26	2.51
No. experiments	4	

Conditions and substrate concentrations were same as in Table II. DNP was .1 μMole/flask.

ported by them was sufficient to produce complete inhibition of fluoroacetate conversion to fluorocitrate. In our system we were unable to show a reactivation of respiration when DNP and fluoroacetate were incubated together, even at higher DNP concentrations.

Summary. In presence of fumarate, rat liver mitochondria responds with citrate accumulation to fluoroacetate poisoning. This response is not sex-dependent. There is a time lag to the inhibitory effect of fluorocompounds on respiration. Ratios of respiration of fluoroacetate and control system of starved and fed animals were in close agreement. Testosterone did not abolish the effect of fluoroacetate on citrate accumulation, whereas progesterone significantly reversed it only on liver mitochondria of male rats. Progesterone, like DNP, when in combination with fluoroacetate, inhibited respiration. In normal nutritional states irradiation does not significantly increase citrate accumulation in the liver mitochondria of male rats.

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Received October 19, 1959. P.S.E.B.M., 1960, v103.

Buffy Coat Cell Migration from Symmetrical Explants in Presence and Absence of Clotted Plasma.* (25537)

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Difficulties most commonly encountered in work concerned with buffy coat cell migration are (a) it is very difficult, if not impossible, to obtain symmetrical and reproducible explants using conventional technics, (b) migration is slow when cells are imbedded in clotted plasma. Our purpose was to develop a simple and reproducible procedure to obviate these difficulties.

The method consists essentially of filling small bore polyethylene tubing with material to be studied and then cutting it into small pieces handled as if they were tissue explants. Intradermic polyethylene tubing formulation PH-F-Adams PE-50† was sterilized by filling

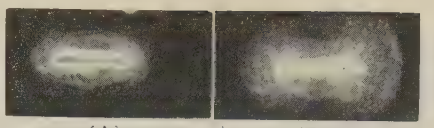
* Supported by grant from Nat. Heart Inst.

† Sterile tubing obtained from Clay Adams.

it with 1:1000 zephiran chloride. After 18 hrs, tubing was washed with several changes of sterile distilled water, cut into 6 inch long pieces then placed in test tubes and dried in incubator at 37°C. Glass chambers were sealed together with petrolatum-paraffin-wax mixture,‡ and sterilized under ultraviolet germicidal tube. Ten ml of heparinized§ blood were placed in siliconed tubes, kept for 2 hrs at room temperature, then centrifuged 10 min at 1500 rpm with radius of 20 cm. Supernatant plasma was discarded, buffy coat harvested, transferred to hematocrit tubes (made from pyrex glass tubing of 6 mm internal diameter) and centrifuged as before. Supernatant plasma was pipetted off and buffy coat transferred to another tube and mixed gently. Tip of plastic tube was dipped into buffy coat cell suspension and filled by slow gentle suction applied with automatic 2 ml syringe through 23 gauge needle. One end of tube was softened with small flame and sealed by crimping tightly with fingers. Plastic tubes were then inserted individually into 6″ long, 16 gauge metal tubes (like those used for filling Winthrop hematocrit) and again centrifuged 10 minutes at 3000 rpm to pack the cells. The sealed tubes were sterilized by immersion in 95% alcohol and then left at room temperature until alcohol evaporated. Tubes were cut aseptically into small pieces about 3.6 mm long containing approximately 1 mm^3 of packed cells. The small pieces were moistened with chick embryo extract and placed in chambers, as if they were tissue explants, in tiny drop of chicken plasma which clotted rapidly and fixed them to the glass. Sealed chamber was then filled with Hanks serum (7 parts Hanks and 3 parts pooled guinea pig serum) or a semisolid Hanks-serum agar medium (HSAM), with 0.5% agar, using syringe and needle. Needle was introduced through one of 2 perforations made with inoculating wire and sealed subsequently. When cell-plasma clot explants were desired, cell-plasma-embryo extract mixture was drawn rapidly into plastic tubing, allowed to clot, sliced and

‡ Petrolatum 0.5 part, paraffin 1 part, wax 3 parts by weight.

§ 5 USP units/ml blood.



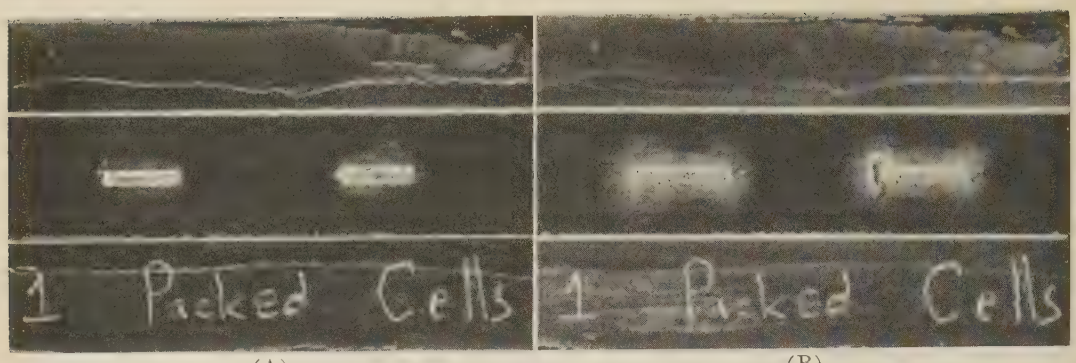
(A) (B)

FIG. 1. Differences in cell migration from buffy coat explants (A) with, and (B) without plasma clot.

explanted to a chamber as described above. Chambers were incubated at 37°C and examined at regular intervals to 18 hrs for cell migration at both ends of tube (Fig. 1-A, B). The cross sectional area of tube, not its length, is the critical factor in migration.

Chambers are made with 3 standard (3 x 1″) microscope slides as follows: One is split lengthwise and strips held ⅜″ apart with spring clips attached to flat-ground metal plate. A second slide is centered on top of strips and 2 drops of melted wax-paraffin-vaseline mixture applied to each side. Plate is heated until mixture flows evenly by capillarity after which plate is cooled and chamber removed. Two plugs about ¼″ long are gradually built-up, in trough over ends of chamber, with melted drops of a paraffin-vaseline (1:2) mixture until raised above level of depression. After solidification, excess is cut flush with razor. Chamber is now ready for washing, drying and ultraviolet light sterilization. Following insertion of explants, a third, sterile slide is placed on top and sealed with wax-paraffin-vaseline mixture.

Results. 1. Cell migration is obtained usually at both ends of tube with both packed cells and cells incorporated into a plasma clot. This enables observer to take 2 readings from a single explant, one at each end. 2. There are individual differences in cell migration with material from apparently normal persons at end of 18 hrs but these were minimized when packed cells in absence of plasma clot were used. 3. Packed cells migrated faster and for longer distances in Hanks serum than did those from plasma clot explants, or than cells incorporated into HSAM (Fig. 2-A, B). 4. Cells remain active for significantly longer time and their phase image shows more contrast when suspended in HSAM than when suspended in plain liquid Hanks-serum medium.



(A) (B)

FIG. 2. Polyethylene tubes with packed cells, without plasma clot inside closed chamber containing Hanks-serum medium (A) before, and (B) after 18 hr incubation at 37°C. Notice migration at both ends.

Migration studies were carried out with buffy coats from 25 blood bank donors with both packed cells and cells incorporated into plasma clot. Three similar explants were used in each instance and readings made, measuring distance from end of tube to advancing front of migrating cells. In Table I, each value represents average of 6 readings. Additional determinations were made in 75 blood bank donors using packed cells without plasma with mean migration of 3.73 mm after 18 hrs. Individual differences in migration are minimized when packed cells without clotted plasma are used. There is a statistical difference in cell migration from tubes with packed cells and tubes containing cells embedded in plasma clot as evidenced by lack of overlapping of corresponding ranges.

TABLE I. Human Buffy Coat Cell Migration after 18 Hr Incubation Using Polyethylene Tube (PE) Technic in Presence and Absence of Plasma Clot.

Cells suspended in chicken plasma clot in PE tube	Cells packed in PE tube by centrifugation in absence of plasma clot	Cells suspended in chicken plasma clot in PE tube	Cells packed in PE tube by centrifugation in absence of plasma clot
(migration in mm)		(migration in mm)	
2.75	3.30	3.25	3.79
2.82	3.27	2.63	4.05
1.80	3.84	1.20	3.86
3.35	3.79	1.92	4.05
1.89	3.39	2.25	3.65
1.59	3.15	2.90	3.68
1.29	3.05	1.76	3.30
1.50	3.84	2.10	3 28
1.71	3.30	1.50	3.56
1.05	4.95	1.05	3.33
2.70	4.20	1.85	3.75
2.78	3.35	2.25	4.05
1.83	5.10		
Mean ± S.D. (mm)		2.07 ±.67	3.72 ±.51
Range		1.05-2.90	3.05-5.10

Cell migration from bone marrow can be studied by this method. Bone marrow can be easily aspirated into the tube, or marrow is placed into small syringe and forced into tubing through Clay-Adams polyethylene tubing syringe adapter of suitable bore. Tubes are centrifuged to pack material and then handled as described above for buffy coat.

Summary. 1. A simple and easily reproducible technic for study of cell migration from explanted buffy coat and bone marrow is described. 2. There are individual differences in cell migration with buffy coat from normal persons at the end of 18 hrs, but these are minimized when packed cells, in absence of plasma clot, are used.

Received October 26, 1959. P.S.E.B.M., 1960, v103.

Effect of Phenylalanine Metabolites on Urinary Excretion of Indole-3-acetic Acid and 5-Hydroxyindole-3-acetic Acid in Man.* (25538)

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Increased urinary excretion of indole-3-acetic acid (IAA), indole-3-lactic acid, and indican, and decreased excretion of 5-hydroxyindole-3-acetic acid (5-HIAA) have been reported for the hereditary metabolic disease phenylketonuria(1-3). However, the relation between the increased concentration of phenylalanine and its metabolites in this disease and the disturbance in tryptophan metabolism has not been adequately explained. It has recently been found that the pyridoxine-requiring enzymes dihydroxyphenylalanine decarboxylase, 5-hydroxytryptophan decarboxylase, and glutamic acid decarboxylase can be inhibited *in vitro* by certain metabolites of phenylalanine(4-6). These findings suggest that the *in vivo* inhibition of tryptophan decarboxylase as well as 5-hydroxytryptophan decarboxylase might possibly underlie the alteration in tryptophan metabolism associated with phenylketonuria. *In vivo* evidence for the reversibility of this type of inhibition was demonstrated when it was shown that urinary 5-HIAA levels could be elevated to normal values in phenylketonurics when placed on low-phenylalanine diets or fed L-tryptophan (7,8), or reduced in patients with carcinoid tumors after ingestion of phenylacetic acid(9). Few data, however, have as yet been presented on the *in vivo* effects of specific phenylalanine metabolites on excretion of urinary indoles in healthy individuals. The present report describes the effect of phenylpyruvic acid, *o*-hydroxyphenylacetic acid, and phenylacetic acid on urinary excretion of IAA and 5-HIAA in normal human subjects.

Methods. The 5 adult males used as subjects were between 22 and 36 years of age. Two-hour control urine samples were taken from the test subjects prior to administration of the test compound. The orally administered dosages were: sodium phenylpyruvate† (20 mg/kg), phenylacetic acid† (20 mg/kg), and *o*-hydroxyphenylacetic acid‡ (2 mg/kg). Urine samples were then collected every 2 hours for the following 6-hour period. The urine samples were immediately assayed for indican (indoxylsulfate), IAA, and phenylpyruvic acid and then frozen for subsequent analysis. The frozen samples were assayed for *o*-hydroxyphenylacetic acid and 5-HIAA within 3 days. Indican was estimated by Parker's modification of the procedure of Askenstedt(10); no attempt was made to quantify these values which are presented as relative figures only. The colorimetric method of Berry and Woolf(11) was used for the phenylpyruvic acid assay, and a modification of the paper-chromatographic procedure of Armstrong *et al.*(12) for the *o*-hydroxyphenylacetic acid assay in which the spots were developed with 2,6-dichloroquinone chlorimide. IAA was measured by the colorimetric method of Weissbach *et al.*(13) and the 5-HIAA by the colorimetric technic of Udenfriend *et al.*(14). Because indolelactic acid is also extractable by the procedure used for the IAA assay, paper chromatograms were run on the urine extract as described by Weissbach *et al.*(13); no increase in indolelactic acid over control values was detectable. Even so, the contribution of indolelactic acid to the IAA values can not be completely discounted. The values for 5-HIAA were corrected for an estimated 85% recovery.

Results. Increases in the urinary excretion of IAA and 5-HIAA usually occurred within the first 2-hour period following administration of a specific phenylalanine metabolite. Increases in IAA excretion were in all cases greater than those of 5-HIAA.

Phenylpyruvic acid administration. Ingestion of phenylpyruvic acid resulted in a

* This investigation was supported in part by research grant from Nat. Inst. of Arthritis and Metab. Dis., N.I.H., U.S.P.H.S.

† Nutritional Biochemicals Corp.

‡ California Corp. for Biochemical Research.

TABLE I. Urinary Excretion of Phenylpyruvic Acid (PPA), *o*-Hydroxyphenylacetic Acid (*o*-HPAA), 5-Hydroxyindoleacetic Acid (5-HIAA), Indoleacetic Acid (IAA), and Indican (IND) after Oral Administration of Sodium Phenylpyruvate (20 mg/kg Body Weight).

Hr after admin.	PPA	*o*-HPAA	IAA	5-HIAA	IND
			(μmoles)		
	Subject A (dose: 8.3 mmoles)				
0	0		1.7		3.6
2	1121		10.6		3.7
4	4.3		8.2		1.7
6	0		6.2		1.1
	Subject B (dose: 10.4 mmoles)				
0	0.		2.3		2.4
2	1456		7.8		1.5
4	14.6		8.1		.9
6	0		3.3		.5
	Subject C (dose: 7.9 mmoles)				
0	0	.7	3.6	2.3	
2	904	12.2	16.6	8.2	
4	17.1	8.7	7.3	2.3	
6	0	4.3	6.6	2.9	

marked increase (3.5- to 6.2-fold) in the urinary excretion of IAA (Table I) and a 3.5-fold increase (one subject) in 5-HIAA. Since phenylacetic acid and possibly *o*-hydroxyphenylacetic acid(15) are derived from phenylpyruvic acid, the response to phenylpyruvic acid loading must be the result of the combined effect of all 3 metabolites. The low recovery (less than 14%) of administered phenylpyruvic acid attests to the efficient metabolism of this compound. No significant alteration in excretion of indican could be detected by the method employed.

TABLE II. Urinary Excretion of *o*-Hydroxyphenylacetic Acid (*o*-HPAA), Indoleacetic Acid (IAA), and 5-Hydroxyindoleacetic Acid (5-HIAA) after Oral Administration of *o*-Hydroxyphenylacetic Acid (2 mg/kg).

Hr after admin.	*o*-HPAA	IAA	5-HIAA
		(μmoles)	
	Subject D (dose: 2.1 mmoles)*		
0	1.5	2.9	1.4
2	814	19.5	2.4
4	503	14.3	4.3
6	425	4.7	1.7
	Subject A (dose: .9 mmole)		
0	.8	2.1	1.3
2	514	15.8	4.1
4	177	12.4	3.0
6	53	4.5	1.6
	Subject E (dose: 1 mmole)		
0		1.7	1.9
2	500	9.4	4.8
4	224	5.9	1.2
6	134	4.8	

* 4.5 mg/kg.

o-Hydroxyphenylacetic acid administration. The high recovery (greater than 83%) of the ingested *o*-hydroxyphenylacetic acid indicates that the alterations in the urinary excretion of IAA and 5-HIAA are due almost entirely to the unmetabolized compound. The sensitivity of tryptophan metabolism to *o*-hydroxyphenylacetic acid is borne out in Table II. Here, although the *o*-hydroxyphenylacetic acid dose was only 1/10 that of the phenylpyruvic acid test, IAA was excreted at higher levels (5.5- to 7.5-fold increase) than those produced by phenylpyruvic acid loading. The 2.5- to 3.1-fold increase in excretion of 5-HIAA was seemingly within the range induced by the phenylpyruvic acid test.

Phenylacetic acid administration. The 2- to about 4-fold increase in IAA (Table III) brought about by phenylacetic acid ingestion appeared to be similar to, or somewhat lower than, the increases resulting from phenylpyruvic acid administration. There is some indication of depression in excretion of 5-HIAA.

TABLE III. Urinary Excretion of *o*-Hydroxyphenylacetic Acid (*o*-HPAA), Indoleacetic Acid (IAA), and 5-Hydroxyindoleacetic Acid (5-HIAA) after Oral Administration of Phenylacetic Acid (20 mg/kg).

Hr after admin.	*o*-HPAA	IAA	5-HIAA
		(μmoles)	
	Subject A (dose: 10 mmoles)		
2	.6	10.3	.8
4	.7	7.0	1.3
6	.7	2.2	.8
	Subject E (dose: 11.5 mmoles)		
0		3.1	1.8
2		6.2	.5
4		3.4	.8
6		4.5	.9

Discussion. If we assume that the same metabolites that inhibit 5-hydroxytryptophan decarboxylase(5) can inhibit tryptophan decarboxylase as well, then the blocks in amine formation as outlined in Fig. 1 can be postulated. That tryptophan undergoes decarboxylation to tryptamine in mammalian tissues is supported by the recent findings of Weissbach *et al.*(13). Inhibition in formation of tryptamine and 5-hydroxytryptamine could have the effect of shunting an increased

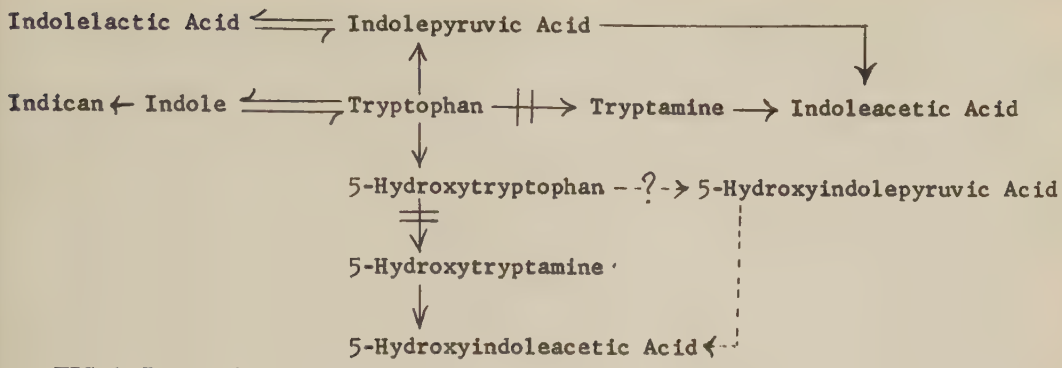

FIG. 1. Intermediary pathways in formation of indoleacetic acid, indolelactic acid, 5-hydroxyindoleacetic acid, and indican from tryptophan.

amount of tryptophan to the transamination route to indolepyruvic acid and IAA (Fig. 1). It is not clear, however, just why indolelactic acid is not also excreted in increased amounts. In this connection, it is also of interest that the increased levels of urinary indican reported in phenylketonurics(2) could not be experimentally induced by the PPA loads administered in the present study.

Because of the lack of sustained stress in the present work, the inhibition picture discussed above, although possibly applicable to the pathological state in phenylketonuria, would not necessarily apply to the results of the present study. An alternate explanation would be that these phenylalanine metabolites have the capacity, either directly or indirectly, to displace bound tryptamine and 5-hydroxytryptamine from tissues. These released amines then undergo conversion by amine oxidases to IAA and 5-HIAA. This could explain the paradox in the present study of an increase in excretion of 5-HIAA rather than the expected suppression resulting from inhibition of 5-hydroxytryptophan decarboxylase; for only when an equilibrium is reached and the tissue amine is depleted would the overall inhibitory effect become apparent. The ability of certain compounds to displace tissue 5-hydroxytryptamine has been attributed to their antimetabolite action(16).

The possibility remains that some 5-HIAA is formed *via* oxidative deamination of 5-hydroxytryptophan to 5-hydroxyindolepyruvic acid. If this route is operative, however, it does not appear to be an effective alternate pathway for the metabolism of 5-hydroxytryptophan. There is as yet no evidence for such a pathway in the mammal.

Summary. 1) Oral administration of phenylpyruvic acid, phenylacetic acid, and *o*-hydroxyphenylacetic acid to human subjects resulted in increase in urinary excretion of indoleacetic acid. A smaller increase in excretion of 5-hydroxyindoleacetic acid followed administration of phenylpyruvic acid and *o*-hydroxyphenylacetic acid. 2) *o*-Hydroxyphenylacetic acid was demonstrated to be more effective than either phenylpyruvic acid or phenylacetic acid in increasing excretion of indoleacetic acid. 3) The relation of these findings to inhibition of tryptophan decarboxylase and 5-hydroxytryptophan decarboxylase is discussed.

The author acknowledges the able technical assistance of Patricia A. Llewellen.

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Received October 30, 1959. P.S.E.B.M., 1960, v103.

Some Characteristics of Proteolytic System of a Marine Bacterial Species.* (25539)

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The desirability of increasing present limited knowledge of bacterial proteolytic enzymes may be inferred from work of previous authors(1-4), some of whom suggested potential usefulness of these enzymes in investigations of protein and enzyme structure and in industrial processes(3,4). Investigations of bacterial proteinases have dealt principally with enzymes from terrestrial species; marine organisms have been largely neglected, even though they have been reported more proteolytic than either soil or fresh water forms(5). In view of established proteolytic ability, marine bacteria appear to offer considerable promise in investigations designed to increase our overall information on bacterial proteolytic enzymes. This report describes characteristics of a proteolytic system of an organism isolated from a marine environment.

Materials and methods. The organism was a marine bacterial species isolated by Merkel and Traganza(6) from the alimentary canal of the marine isopod, *Limnoria,* and reported by them to be highly proteolytic.† Stock cultures were grown on slants consisting of 1% peptone and 2% agar in artificial sea water, and were transferred at monthly intervals. A transfer was made from stock culture to a slant identical to stock medium, except that artificial sea water diluted 1:10 with distilled water was used. After 12 to 24 hours incubation, a loop transfer was made to 50 ml Erlenmeyer flask containing 6 ml of growth medium (Table I); this subculture was incubated 12 to 24 hr before use to inoculate 1 liter of growth medium. Incubation was at 25°C with shaking. After 54 to 56 hours incubation, cells were removed by centrifugation and passage through a Seitz filter; the resulting culture filtrate was used as source of enzyme. One ml of enzyme solution was incubated at 37°C with 5 ml of denatured hemoglobin substrate.‡ At end of desired incubation time, reaction was stopped and residual protein precipitated by addition of 10 ml of 5% trichloroacetic acid. The mixture was filtered through Whatman No. 3 paper, and determination of acid-soluble split products was made by the method of Northrup *et al.* (7) by reading at 280 mμ in Beckman Model DU Spectrophotometer. Enzyme concentration and time of incubation were always chosen so that activity was proportional to

* Supported, in part, by grants from Nat. Inst. Allergy and Infect. Diseases and from Robert A. Welch Fn.

† We are indebted to Dr. Joseph R. Merkel and Eugene D. Traganza for this culture, and for information on its characteristics prior to publication. Morphological, physiological, and biochemical characteristics of the organism are to be published by these workers.

‡ Prepared by suspending 5 g salt-free lyophilized hemoglobin (Mann Research Labs.) in 80 ml of water, adding 8 g urea, and incubating at 37° C for 45 to 60 minutes. Ten g urea and 125 ml of 0.2 M phosphate buffer (pH 8.0) were then added. Other pH values were employed where indicated.

TABLE I. Composition of Growth Medium.

Component	Amount/l
Peptone	10.0 g
NaCl	8.12
$MgCl_2 \cdot 6H_2O$	4.22
K_2HPO_4	1.0
KH_2PO_4	1.0
TRIS*	1.0
$MgSO_4 \cdot 7H_2O$	.4
Trace mineral solution†	5.0 ml

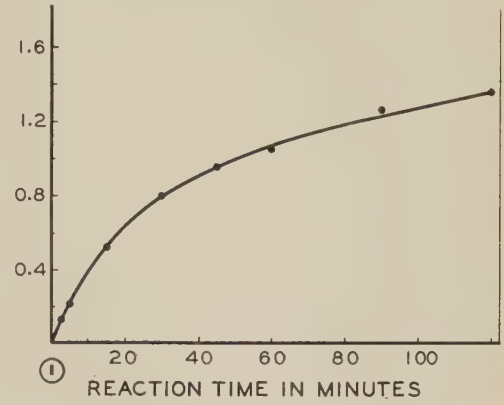
* Trishydroxymethylamino methane.

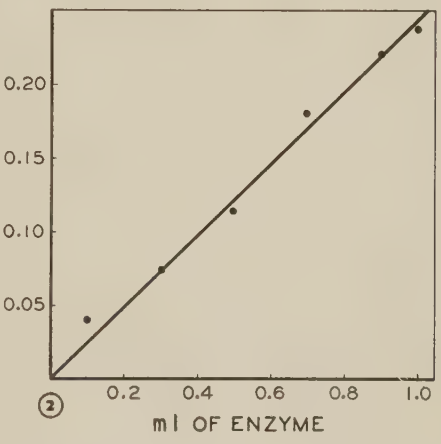
† Components, and concentrations in g/l: Ethylenediaminetetraacetic acid (tetrasodium salt), 1; H_3BO_3, 1.14; $FeCl_3 \cdot 6H_2O$, .049; $FeSO_4 \cdot 7H_2O$, 4; $MnSO_4 \cdot H_2O$, 3.08; $ZnSO_4 \cdot 7H_2O$, .022; $CuSO_4 \cdot 5H_2O$, .00016; $CoSO_4 \cdot 7H_2O$, .00048.

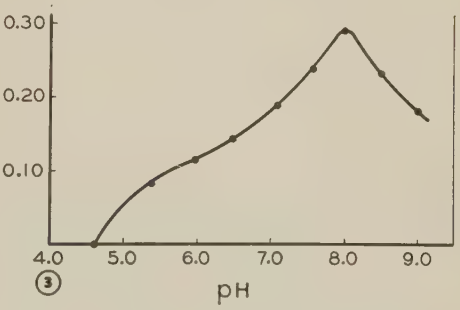
amount of enzyme and to time of incubation. Five minute incubation was commonly used; however, assays were frequently performed at several different time values so that activity could be plotted as a function of time. Appropriate blanks were used in all experiments.

Results. Culture filtrates prepared as described were so active proteolytically that it was necessary to dilute them 1:10 to obtain accurate assays at 5 minutes incubation. Filtrates could be frozen and stored several weeks without appreciable loss of activity. Our experiments did not permit us to determine whether proteinase is truly extracellular, or whether it appears in growth medium through autolysis of cells(2). At short periods of incubation, rate of proteolysis was proportional to reaction time (Fig. 1) and to enzyme concentration (Fig. 2). Other experiments showed that rate of reaction was affected by substrate concentration, rising rapidly to maximum value at 8 mg of hemoglobin/ml, and decreasing slightly at higher levels. Similar behavior has been reported for other bacterial proteinases(8,9).

Optimum pH for enzyme activity was determined by buffering samples of substrate at desired pH values with phosphate buffer. These experiments revealed a single, sharply defined optimum at pH 8 (Fig. 3). This is slightly more alkaline than the optima of some extracellular bacterial proteinases previously reported(3,9), but is distinctly more acidic than that of subtilisin(10). It is perhaps significant that the optimum pH for the proteinase system of the marine organism coincides almost exactly with the pH of sea water(11). Effects of pH on enzyme stability were tested by determining activity at different incubation times (up to 30 min) in samples of substrate adjusted to pH values of 7.0,







All ordinates: Optical density at 280 mμ.

FIG. 1. Effect of reaction time on hydrolysis of hemoglobin by culture filtrate of the marine bacterium.

FIG. 2. Effect of enzyme concentration on proteolysis. Incubation time, 5 min.

FIG. 3. Influence of pH on activity of proteolytic system. Samples of substrate were buffered with phosphate buffer at values shown.

7.5, 8.0, 8.5, and 9.0. The enzyme system was stable at all of these pH values at incubation times tested, and comparative slopes of the activity plots were identical to those which one would predict from data in Fig. 3.

Effects of temperature on enzyme activity. Six temperatures of incubation from 17°C to 55°C were tested at incubation times ranging from 5 to 60 minutes. Activity increased with temperature through 48°C, but declined somewhat at 55°C, indicating partial inactivation of the proteinase system at the highest temperature. From these data, minimum energy of activation for the proteinase system was calculated to be about 8300 cal. To determine effects of temperature on stability of the enzyme system, samples of the culture filtrate were heated at 70°C for varying times, chilled instantly in ice water, then assayed for remaining enzymatic activity. Five minute exposure at 70°C reduced activity to 87% of original, 7.5 minutes to 50%, and 13 minutes of heating virtually destroyed enzymatic activity.

Effects of activators and inhibitors. Prolonged dialysis of culture filtrate against 0.01 M phosphate buffer (pH 8) resulted in sharp reduction of enzymatic activity, indicating a requirement for one or more activators normally present in the growth medium. Approximately 80% of original activity was restored by rendering the dialyzed enzyme 0.005 M with respect to Co^{++} ions. Similar activating effects of Co^{++} ions on bacterial proteinases have been observed by Weil and Kocholaty (12), and by Hunt and Moore(13). Activity was restored to about 60% of the original by adding Zn^{++} ions to a concentration of 0.0005 M, but higher levels reduced activity. Undialyzed enzyme was inhibited by 0.005 M concentrations of $HgCl_2$, $FeCl_3$, $NiCl_2$, and $CuSO_4$. No decrease in activity occurred when *p*-chloromercuribenzoate was added, indicating that sulfhydryl bonds are not required for activity. Furthermore, reducing agents (cysteine, glutathione, KCN) produced marked reduction of activity at a concentration of 0.005 M, thus indicating a necessity for disulfide linkages. Previous investigators have also observed some inhibition in activity when reducing agents were added to proteolytic enzymes of aerobic bacteria(14,15). Damodaran *et al.*(3) reported that reducing agents were without effect on the proteinase of *Bacillus licheniformis,* while Hunt and Moore(13) found inhibition by both *p*-chloromercuribenzoate and cysteine on a proteinase from a facultatively aerobic rod.

Summary. Filtrates from pure cultures of a marine bacterial species contain a proteolytic enzyme which rapidly hydrolyzes denatured hemoglobin. Enzymatic activity after 54 to 56 hours of growth is so high that culture filtrates must be diluted for accurate assay even with short reaction periods. The enzyme system has optimum activity at pH 8, is activated by Co^{++} and Zn^{++} ions, and is inhibited by Fe^{+++}, Hg^{++}, and Cu^{++} ions. Disulfide linkages appear to be required for activity, since reducing agents inhibit activity and *p*-chloromercuribenzoate is without effect. The enzyme is highly active at 48°C during incubation periods to 40 minutes, but temperatures above 50°C reduce activity even at shorter incubation periods. Heating at 70°C for 7.5 minutes before assay reduces enzyme activity to one-half of original.

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Received November 4, 1959. P.S.E.B.M., 1960, v103.

Spontaneous Activity in Gamma Efferents of a Deafferented Spinal Cord Segment. (25540)

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Isolation of spinal cord segment from descending and afferent impulses leads in puppies(1), cats(2) and monkeys(3) to contractile inactivity of parts innervated by the segment. Even the spinal dogfish, which has incessant spontaneous swimming movements when dorsal roots are intact, becomes quiet when sufficient roots are cut(4). Absence of overt contractions, of course, indicates that there is no firing of the large alpha motoneurons which innervate extrafusal muscle fibers. Not eliminated, however, is the possibility of unrevealed activity in the small fusimotor neurons or gamma efferents leading to intrafusal fibers of muscle spindles. Under many conditions of physiological and experimental stimulation, spindle loops may be in vigorous fluctuating activity without alpha motoneuron participation(5). The firing of these fusimotor neurons can be influenced from both segmental and supraspinal levels(6). Bilateral exclusion of dorsal root inflow does not extinguish unevoked activity in these neurons in animals with intact supraspinal connections(5); nor does interruption of descending pathways prevent continuing activity if segmental inflow is intact(7). In our experiments, both segmental afferent and descending influences have been excluded from the cat's lumbosacral cord to see if activity in fusimotor fibers may be fully independent. Evidence of spontaneous activity of fusimotor neurons under these conditions has been obtained in acute and chronic preparations.

Methods. The lumbosacral segment of spinal cord in 16 cats was isolated from impinging nervous impulses by transecting at L_1 and cutting all dorsal roots to the segment. Transection in acute experiments was preceded by freezing of the cord at immediate site of sectioning; in chronic preparations, either the same method was used, or dura was left intact and a cut made between ligatures tied around the dural sac. Dorsal roots were clamped with silver clips and cut extradurally if deafferentation was performed at time of initial operation, or if left to terminal experiment, the roots were cut along line of their entrance into the cord, at which level it was generally possible to spare major radicular vessels. When cutting extradurally, ventral roots from about S_3 caudalward also had to be sacrificed. Close nursing care involving treatment with antibiotics, forced feeding, and bladder expression was given to chronic animals over 2-30 days. At time of terminal observation, cats were electrolytically decerebrated, the lumbosacral cord bathed in mineral oil maintained within 1° of normal body temperature by thermistor-controlled radiant heat, and observations begun 2-6 hours after surgery was completed. Potentials were detected by suspending fine filaments of either ventral or dorsal roots on submerged silver electrodes. When dorsal root fibers were monitored, the hind leg was denervated except for the nerve to triceps surae, and units were identified as spindle afferents by presence of a pause in discharge during induced twitch. Checks were routinely made for completeness of deafferentation by inspection for reflex responses upon stimulation of the skin, through search for afferent activity in dorsal roots in preparations with extradurally cut roots, and by postmortem inspection of root and cord lesions.

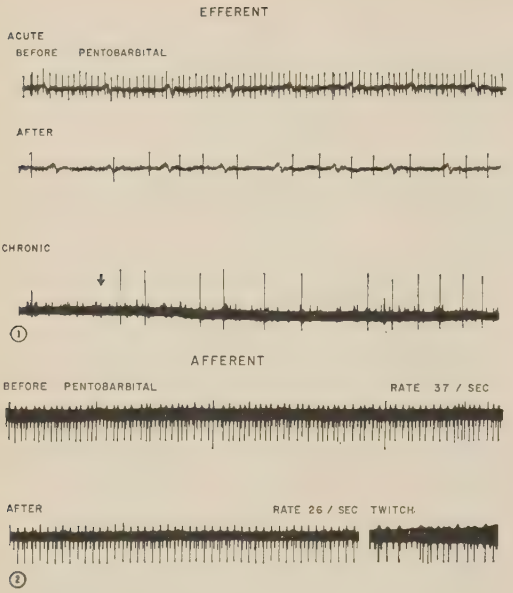

FIG. 1. A. Efferent unit from S_1 VR of cat in which cord was transected at L_2 and lumbosacral segment deafferented 5 hr previously. Midbrain decerebration. Discharge is shown before and after slowing of the unit by administration of intrav. pentobarbital. This unit had been monitored for one hr before the upper trace was taken. B. Efferent spikes in S_1 VR filament 8 days after transection and deafferentation. Arrow indicates that dorsum of cord was touched to excite large alpha spikes seen contrasting with the background of gamma efferent discharge.

FIG. 2. Unit in S_1 DR of acute preparation before and after pentobarbital. Discharge dropped from 37/sec. to 26/sec. and became more regular. Twitch response at lower right identifies unit as a spindle afferent in the triceps surae muscle. Muscle tension remained unchanged.

Results. Presence of activity in ventral roots. Total absence of spontaneous phasic or tonic contractions and marked degree of atrophy in those muscles innervated by the chronic deafferented spinal cord segment of these adult cats accords with similar observations from cats(2), puppies(1), and monkeys (3). Only when pressure was applied along the mid-dorsal scar would movements of hind limbs occur, probably as a result of direct irritation of the cord(1). This did demonstrate that the cord was viable, and that alpha motoneurons could be aroused to fire. Despite absence upon simple observation of spontaneous movements in hindquarters of chronic deafferented spinal cats, these animals after surgical preparation for recording, demonstrated sustained spiking discharge in ventral roots of the isolated cord segment (Fig. 1, lowermost trace). Single units, firing at rates of 6-25/sec., and with fair regularity, could be followed for over a half hour. Administration of ether or intravenous injection of pentobarbital caused slowing and final silencing of this discharge.

In 2 animals, to lessen the chance for occurrence of an episode of cord ischemia, cutting of dorsal roots was delayed until terminal experiment 7 or 10 days later. Several acutely transected and deafferented preparations were also examined to avoid denervation sensitization (Fig. 1, upper traces). Ventral roots in both of these types of preparation showed a discharge similar to that of chronically deafferented spinal preparation.

Identification of activity. Spontaneously discharging spikes were small contrasted to other spikes which could be aroused to fire by electrical or mechanical stimulation of surface of cord (Fig. 1, lowermost trace). This, together with absence of overt contractile activity suggested that small spikes arose in fusimotor fibers leading to muscle spindles, and the large ones of temporary appearance in motoneurons innervating extrafusal muscle. Continuing action in fusimotor fibers to a spindle should 1) enhance the rate of afferent discharge from that spindle, and 2) induce irregularity in its discharge(5). Fig. 2 illustrates these effects in discharge from a spindle unit in the medial gastrocnemius muscle of a cat in which the cord and dorsal roots were severed 2 hours before observations. In the upper trace, the afferent is firing at a rate of 37/sec., and wavelike irregularity of the intervals is evident. The lower record, taken after intravenous administration of an anesthetic dose of pentobarbital, shows a changed picture in which the rate is 26/sec., and the intervals are regular. As pentobarbital has only minor direct effect on spindle afferents, these changes were due to elimination of tonic discharge in fusimotor fibers to that spindle.

Discussion. The question arises whether the activity detected in these preparations is an attribute of normal cord function or an artifact admitted by severe conditions of the

experiment. Temporary ischemia of the spinal cord, for example, might have led to loss of interneurons, decrease in net inhibition, and resultant sustained discharge of motoneurons including gamma units(8). However, in our chronic animals, ischemic rigidity was avoided, since arteries entering along ventral roots were undisturbed, those along dorsal roots were spared and, in some preparations, compensatory adjustments in circulation were favored by making the transection and deafferentation at different times. Lesser effects due to relative ischemia of long duration, or to sensitization of denervated neurons are excluded in animals prepared acutely.

The flurry of injury discharge which follows procedure of isolating a ventral root filament may dwindle to a slow discharge that must be distinguished from spontaneous activity, particularly since myelinated axons of small size are more prone to demonstrate injury potentials(9). That units were not accepted as truly spontaneous unless firing was sustained for over 5 minutes, and that some were followed for periods to an hour is reassuring. Moreover, injury potentials in ventral roots due to handling were eliminated as a factor in experiments in which afferent units were monitored. Triggering of cord activity from hyperexcitable tissue lying adjacent to cord transection is a further possibility.

The volume of discharge heard in ventral rootlets of these preparations is not as great as the similarly pure fusimotor activity in roots leading from deafferented levels of the cord in decerebrate cats. This and the observation that silent units of probable fusimotor nature can be aroused to activity with stimulation of the isolated cord segment suggests that only a minor portion of available gamma units is spontaneously active.

Summary. Presence of spiking discharge in fibers of ventral roots leading from lumbosacral cord completely deafferented and transected at the L_1 level is described. This activity was noted in adult cats either chronically or acutely prepared. On the bases of the small relative height of these spikes and the demonstration in afferent discharge from muscle spindles of tonic fusimotor influence, it is concluded that ventral root activity arises in gamma efferent fibers. Probably only a minor portion of gamma fibers present is participating.

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Received November 16, 1959. P.S.E.B.M., 1960, v103.

Experimental Shigella Infections. III. Sensitivity of Normal, Starved and Carbon Tetrachloride Treated Guinea Pigs to Endotoxin. (25541)

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(Introduced by J. P. Ransom)
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Hartley strain guinea pigs may be rendered susceptible to a fatal enteric infection with a strain of *Shigella flexneri* 2a if they are either deprived of food for 4 days prior to challenge or injected subcutaneously with carbon tetrachloride 24 to 48 hours before oral administration of viable bacteria(1,2). The mechanism of this increased susceptibility is not clear.

Certainly some alteration of the animal must occur to allow the dysentery bacilli to multiply. In addition, it is conceivable that death results because pretreatment also might render the animal more susceptible to toxic products elaborated by the multiplying dysentery bacilli.

One toxic product of the dysentery bacillus is its endotoxin or somatic antigen. This communication presents results of experiments comparing susceptibility of normal, starved and carbon tetrachloride treated guinea pigs to intravenously injected endotoxin, and attempts to relate differences in susceptibility to *in vivo* distribution of endotoxin.

Material and methods. Hartley or Walter Reed strain guinea pigs weighing 300 to 400 g were used in all experiments. Those which were starved were deprived of food for 4 days before intravenous administration of endotoxin. Carbon tetrachloride treatment consisted of injecting 0.15 ml of this material subcutaneously usually 24 hours before endotoxin was injected intravenously. Animals were observed for 96 hours, and the LD_{50} calculated by the method of Miller and Tainter (3).

Endotoxin of the Boivin type was prepared from *S. flexneri* 2a strain 2457 which was used in our previous work(1,2). The toxin was extracted from acetone treated cells with trichloroacetic acid and further purified by ethyl alcohol-salt (NaCl) fractionation(4). Extracted material was dissolved in physiological saline to a final concentration of 2.64 mg/ml. This solution contained 0.05 mg N_2/ml. In some experiments Cr^{51} labeled endotoxin was employed(5) and distribution of Cr^{51} in organs of animals determined by procedures previously described(6).

Results. Pooled results of experiments comparing susceptibility of normal, starved and carbon tetrachloride treated guinea pigs to intravenously administered *S. flexneri* 2a endotoxin are summarized in Table I. Normal guinea pigs were quite resistant, LD_{50} being in excess of 1320 μg of endotoxin. In contrast the sensitivity of animals was greatly increased following either starvation or pretreatment with CCl_4. Normal and starved animals succumbing to endotoxin usually did so 24 to 48 hours following administration, whereas carbon tetrachloride treated guinea pigs succumbing to endotoxin usually expired 6 hours to 12 hours following injection.

TABLE I. Mortality Following Intravenous Administration of Bacterial Endotoxin to Normal, Starved and Carbon Tetrachloride Treated Hartley Strain Guinea Pigs.*

Endotoxin dose (μg)	Treatment Normal†	CCl_4	Starved
1320	10/25		5/ 5
264	3/25	8/ 8	6/10
52.8	0/25	34/39	3/10
10.6		13/23	2/10
2.1		13/30	
.4		0/15	
LD_{50}	2500 μg	4.0 μg	52.8 μg
S.E.	1766 μg	3.5 μg	26.4 μg

* Starved animals were deprived of food 4 days prior to administration of the endotoxin; CCl_4 treated animals received 0.15 ml of this drug subcut. 30 hr prior to administration of endotoxin.
† Deaths/Total.

An experiment was next conducted to determine the effect of time of administration of carbon tetrachloride on susceptibility of guinea pigs. Animals of the Walter Reed strain were employed, and at the time this experiment was carried out, carbon tetrachloride treated animals of this strain were more sensitive to endotoxin than the Hartley strain animal. Thus a dose of approximately 10 μg sufficed to kill virtually all treated animals. Results of this study show that pretreatment with carbon tetrachloride was effective in decreasing resistance to endotoxin when administered 24, 48 or 72 hours before the toxin (100, 100 and 70% respectively of the animals in these groups died), but had little effect when given at the same time or 1 hour or 4 hours before endotoxin challenge (0, 10 and 10% respectively of the animals in these groups died).

Since carbon tetrachloride treated guinea pigs are more susceptible to endotoxin than are normal animals, experiments were conducted to determine if differences in distribution of endotoxin exist in treated as compared to normal animals. Preliminary experiments in normal animals indicated that most Cr^{51} bound to endotoxin was in the liver and remained there for long periods while free Cr^{51}

Cl_3 was more generally distributed and appeared early in the urine in considerable amounts. A dose of 52.8 μg of Cr^{51} labeled endotoxin was employed because normal animals survive this level while a large number of pretreated guinea pigs do not. Carbon tetrachloride treated and normal animals were paired prior to injection with endotoxin. When carbon tetrachloride treated animals expired their counterparts were sacrificed and distribution of the chromium label in their organs was determined. The results show that no apparent differences in distribution of the chromium label in the liver, spleen or lung were observed in the normal group which survived or treated animals which succumbed. The mean endotoxin content of each of these organs was 6.4, 24.3 and 3.0 μg respectively. Radioactivity of brain, cecum, large intestine, small intestine, kidneys and blood was very small and well within counting error. Additional experiments indicated that no differences in distribution of labeled endotoxin could be detected one hour after its intravenous administration to normal or carbon tetrachloride treated guinea pigs. In addition rate of clearance of the endotoxin from the blood of the 2 types of animals did not differ. The mean endotoxin content found in blood 1, 2, 3 and 5 hours following the intravenous inoculation of 200 μg was 6.2, 5.6, 4.2 and 3.8 μg respectively.

Discussion. Our results show that carbon tetrachloride treated or starved guinea pigs are more susceptible to intravenously injected endotoxin than are normal animals. Liver damage is the major change caused by these treatments. Starvation brings about a fatty metamorphosis while injection of carbon tetrachloride causes a central lobular necrosis in addition to fatty metamorphosis(1,2).

The short period of time required for the carbon tetrachloride treated animals to succumb is reminiscent of the results of McLean and Weil(7) who found that dogs might die in 2 hours following intravenous injection of endotoxin if the liver was bypassed by an Eck's fistula. The possibility must be considered that the carbon tetrachloride sufficiently damaged the liver to render it ineffective thus producing, as far as its function with respect to endotoxin is concerned, what amounts to an Eck's fistula. That the damaged liver trapped as much Cr^{51} as the normal liver might be used as a legitimate argument against this explanation. However, if we assume that the Cr^{51} label represents endotoxin—and we agree that this can be only an asumption (Braude *et al.*(5))—we could postulate that while the damaged liver could remove the endotoxin from the blood stream, it could no longer render it pharmacologically inactive.

It is not known what role this increased susceptibility to endotoxin has in rendering starved or carbon tetrachloride treated guinea pigs susceptible to a fatal enteric infection with *Shigella flexneri.* We have not been able to demonstrate this material in the blood stream or organs of animals experimentally infected with this organism, but it may be that the available technics are not sufficiently sensitive to detect it. Thus at present we may only speculate that decrease in resistance to this toxic product may be a factor which brings about death of the infected guinea pig.

Summary. Pretreatment of Hartley strain guinea pigs either by subcutaneous injection with carbon tetrachloride or by 4 day period of starvation renders them more susceptible to intravenously administered endotoxin. The LD_{50} for normal animals is in excess of 1300 μg; for starved animals 52.8 μg and for carbon tetrachloride treated animals 4.2 μg. Distribution of Cr^{51} label of Cr^{51} tagged endotoxin in organs of normal animals which survive intravenous dose of 52 μg of endotoxin is the same as that in carbon tetrachloride treated animals which succumb following this challenge.

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Received November 20, 1959. P.S.E.B.M., 1960, v103.

Radiofluoride Distribution in Tissues of Normal and Nephrectomized Rats.* (25542)

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The beneficial effect of the fluoride ion upon incidence of dental caries has stimulated interest in the metabolism of fluoride both in calcified and in non-calcified tissues. The kidney has been of particular interest because it is the major route of excretion of absorbed fluoride. The amount of fluoride in non-calcified tissues has been difficult to evaluate analytically because it is found in minute quantities (micromoles/kg). However, development of the crystal scintillation counter has allowed application of radiotracer technics to certain facets of soft tissue fluoride metabolism. The most useful radioisotope of fluoride is F^{18} which is a positron emitter with a half-life of 109.7 minutes(1).

Procedure. Each of 4 male rats (A-D) was given a 1 ml intraperitoneal injection of radiofluoride in a saline solution containing 0.2 ppm stable fluoride. Two other animals (E,F) were each given a 2 ml injection of the solution. Three of the animals (A,C,E), were heminephrectomized 10 days before the experiment, and the remaining kidney was removed on the morning of the experiment. Each animal was sacrificed 80 minutes after injection. The soft tissues, digested to solution in 5 N NaOH on a steam bath, were diluted to a 25 ml volume. The humeri were dissolved by shaking them in dilute HCl. Radiofluoride contents of the tissues were determined by using a well-type crystal scintillation counter to measure radioactivity in a 10 ml aliquot of the dissolved tissue. These measurements were made over 8-minute periods and were corrected for background count, volume and radioactive decay to one point in time. Chloride contents were determined by the wet ash procedure described by Caster, MacDonald and Armstrong(2). Water contents of the fresh tissues were evaluated by loss in weight on drying at 100°C.

Results. Table I presents in 3 ways the mean radiofluoride concentrations found in the soft tissues of normal and nephrectomized animals. These data (Columns II and III) show an elevation of the absolute radiofluoride concentration in the tissues of the animals deprived of kidney function. However, no marked change in the pattern of soft tissue distribution of fluoride occurred as a result of nephrectomy. This fact is best seen from Table II in which radiofluoride concentration in the water of the soft tissues is compared with that of the plasma water. This ratio of fluoride concentration does not appear to differ in normal and nephrectomized animals. The tissues can be divided (Table II) into 4 groups: 1) Brain tissue had lowest relative radiofluoride concentration. 2) Tail tendon was the only soft tissue which had a radiofluoride concentration greater than plasma water. 3) Heart muscle and skeletal muscle had a radiofluoride concentration close to one-half the concentration present in plasma water. 4) Skin, liver and testicular tissue showed a range of concentrations, when compared to plasma water concentrations, with a high of 0.90 for skin tissue water and a low of 0.68 for liver tissue water.

Table I also gives tissue concentrations of radiofluoride in counts per microequivalent

* This study supported by grant from U.S.P.H.S. The authors thank the Linac Staff of Physics Dept. for help in preparation of the radiofluoride.

† U.S.P.H.S. Post-Doctoral Research Fellow.

TABLE I. Mean Radiofluoride Concentrations in Selected Tissues of Normal and Nephrectomized Rats.

I	II		III		IV		V	
	F^{18} cts/g tissue (wet wt)		F^{18} cts/g tissue H_2O		F^{18} cts/μeq Cl		F^{18} cts/μeq Cl in tissue / F^{18} cts/μeq Cl in plasma	
Tissue	Normal	Nephr.	Normal	Nephr.	Normal	Nephr.	Normal	Nephr.
Plasma	8,902	11,275	9,624	12,189	78	101	1.0	1.0
Muscle	3,661	5,982	4,882	6,710	223	328	2.9	3.3
Liver	4,565	5,013	6,360	8,414	165	287	2.1	2.9
Tendon	11,087	13,502	18,098	21,785	165	186	2.1	1.9
Heart	3,625	4,098	4,696	5,308	108	122	1.4	1.2
Testes	5,539	4,600*	7,614	5,921*	103	84*	1.3	.9*
Skin	5,160	6,828	8,703	10,472	83	116	1.1	1.2
Brain	1,199	2,198	1,537	2,819	34	58	.4	.6

* Lower radiofluoride concentrations in testes of nephrectomized animals were a result of the operative procedure which compromised testicular blood flow because of the anatomical relationships of renal and testicular vasculature(3).

of chloride. In Column V ratios of the radiofluoride concentrations in the tissues, so expressed, to that of the plasma is presented. When radiofluoride contents of the tissues are expressed thus, *i.e.*, in comparison with the predominantly extracellular chloride anion, these ratios are a measure of the comparative magnitude of fluid volumes through which the ions are distributed. On the premise that chloride is located in extracellular fluid a ratio greater than one indicates penetration of fluoride into the intracellular fluid. This result was most conspicuous in skeletal muscle; also, in tendon and liver the ratio was greater than one. Skin, heart muscle and testicular tissues showed values of radiofluoride per unit of chloride which were close to that of plasma. Brain tissue had the lowest ratio, always less than one. This finding indicates a very low fluoride concentration in brain or an efficient barrier to migration and exchange of fluoride.

The soft tissues and plasma of the nephrectomized animals showed only a slight increase in fluoride concentration without any marked change in pattern of fluoride distribution among the tissues in spite of the fact that the nephrectomized animals, in contrast to the normal animals, were prevented from disposing of some fluoride in the urine. The extra radioisotope in the nephrectomized animals was sequestered in the skeleton (Table III) with only moderate increases in amounts present in the soft tissues. The results with nephrectomized animals E and F are a good example of this process since these animals received a double dose of fluoride.

Discussion. Previous work has shown that peak radiofluoride concentrations in soft tissues occur well within 80 minutes after a single dose of the radioisotope(4). Therefore, this period of time is adequate to obtain maximum ionic penetration. Assuming that both chloride and fluoride ions are not concentrated

TABLE II. Ratio of Counts per Minute per ml of Water in Tissue Sample Divided by Counts per Minute per ml of Plasma Water..

	Normal B	Nephr. A	Normal D	Nephr. C	Normal F	Nephr. E	Normal Mean	Nephr. Mean
Plasma	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Tendon	2.04	1.47	2.11	2.13	1.43	1.76	1.86	1.78
Skin	.85	.83	1.03	.88	.83	.86	.90	.86
Testes	.82	.52*	.58	.45*	.97	.48*	.79	.48*
Liver	.69	.56	.60	.74	.74	.77	.68	.69
Muscle	.44	.45	.54	.61	.56	.60	.51	.55
Heart	.49	.43	.47	.46	.50	.41	.49	.43
Brain	.20	.23	.14	.32	.13	.18	.17	.23

* See footnote to Table I.

TABLE III. Radiofluoride Contents of Rat Humeri.

Normal animals	B	D	F
F^{18} cts/min./g humerus (wet wt)	311,762	425,342	376,572
Nephrectomized animals	A	C	E
F^{18} cts/min./g humerus (wet wt)	523,538	459,377	916,589

at sites such as cell membranes then relative amounts of these ions found in a tissue is a measure of the fluid volume available to the ion in the tissue.

The sequestration of fluoride by skeletal structures is well known. It is also well known that tendonous structures can be sites of deposition for calcified mineral. It is possible that the unusual concentration of fluoride in the tendon is associated with this same phenomenon. However, chloride concentrations in tendon water are also high and it is possible that the relatively high fluoride and chloride concentrations in tendon water are due to a loss of water through evaporation during exposure and dissection of the thread-like strands of tendon.

Summary. 1) Radiofluoride given by intraperitoneal injection was absorbed and distributed throughout fluid compartments of the rat. It was found in concentrations exceeding that of plasma in the skeleton and in the tail tendon. In all other soft tissues examined, concentration in tissue water was some fraction of plasma concentration. Tissue concentrations in nephrectomized animals were increased but the pattern of distribution was not changed. The skeleton sequestered the bulk of the radiofluoride which would have been excreted by the kidneys. 2) The radiofluoride content of tissues expressed in terms of chloride content of the tissues varied with the nature of the tissue. It was lowest in the brain, highest in skeletal muscle, and was increased in most tissues of nephrectomized animals. The soft tissue fluid compartment volumes are not the same for the 2 halogen anions fluoride and chloride.

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Received November 30, 1959. P.S.E.B.M., 1960, v103.

Studies in Leukemia: XIV. Effect of Human Serum on Development of Leukemia in AKR Mice.† (25543)

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An antiserum that will prevent induction of leukemia in C3H and Swiss mice can be produced in rabbits by using cell-free filtrates made from the brains of leukemic mice or patients who died of leukemia(1). Experiments were undertaken to ascertain whether a similar antiserum could be prepared in human volunteers. Acceleration of development of leukemia in the AKR mouse by inoculation of cell-free filtrate from human brain(2) served as the experimental model.

Materials and methods. Equal parts of the brains of 4 patients who died of acute leukemia (2 lymphoblastic, one myeloblastic, one monoblastic) were pooled, and a 1.0% suspension was prepared. A cell-free filtrate was made of this as previously described(3). The filtrate was rapidly frozen and stored at

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† Aided by grants from Olivia Sue Dvore and Edward Friedman Leukemia Fns. and Am. Blood Research Soc.

TABLE I. The Protective Effect of Serum Obtained from Immunized Human Volunteers, Contrasted to the Effect of Normal Serum and Untreated Controls. In these experiments AKR mice were challenged with leukemic cell-free brain filtrate.

Exp.	Human antiserum	Normal human serum	Untreated
	%	%	%
Feb. 21, 1959	10/15 (66)	14/15*(93)	10/10 (100)
Mar. 12, "	9/15 (60)	11/15 (73)	8/10 (80)
Apr. 28, "	7/15 (47)	15/15 (100)	10/10 (100)
Totals	26/45 (57)	40/45 (88)	28/30 (93)

* No. developed leukemia/No. inj.
Chi square = 11.13. P = .001.

–65°C. Fourteen male volunteers were injected with aliquots of the filtrate (0.1 cc intracutaneously and 0.9 cc subcutaneously) 4 times at weekly intervals. Four weeks after last injection 250 cc of blood were drawn from each volunteer. Seven days later another dose of filtrate was administered and after 6 weeks an additional 500 cc of blood were drawn. The sera were pooled under sterile conditions. This serum will be referred to as *human antiserum*. For purposes of control, pooled *normal human serum* was used.‡ This serum was obtained at approximately the same time from male donors of similar age as the volunteers. Both groups of sera were processed in an identical fashion, and by the same group.

The cell-free filtrates used for challenging the mice were prepared from a 0.25% suspension of a pool of brains obtained from 4 other patients who died of acute leukemia (2 myeloblastic, one monoblastic, one stem cell). Female AKR mice§ between 10 and 12 weeks of age were injected with 0.5 cc of this cell-free filtrate intraperitoneally. The prophylactic inoculations consisted of intraperitoneal injection of 0.5 cc of serum the day before challenge, day of challenge, and day after challenge.

Results. Three experiments were performed and are summarized in Table I. Significant protection is afforded by the human antiserum whereas that afforded by the normal serum is insignificant in all of the 3 experiments.

The results of challenging the mice with various dilutions of the filtrate are shown in Table II. In this experiment the incidence of leukemia was also significantly reduced in the group treated with human antiserum, whereas normal human serum did not significantly affect the results.

TABLE II. The Results of Challenging Mice with Various Dilutions of Active Leukemic Cell-Free Brain Filtrate.

Conc. of leukemic filtrate, %	Human antiserum	Normal human serum	Untreated
	%	%	%
.25	7/15 (46)	15/15*(100)	10/10 (100)
.025	10/25 (40)	19/25 (76)	7/10 (70)
.0025	3/15 (20)	9/15 (60)	5/10 (50)

* No. developed leukemia/No. inj.

Sera were obtained from the volunteers before immunization. These did not afford significant protection to the challenged mice. One of the volunteers was injected with physiologic saline solution instead of leukemic cell-free brain filtrate. His serum failed to show a protective effect.

Summary. Human volunteers were inoculated with a cell-free filtrate prepared from a pool of brains obtained from patients who died of acute leukemia. The antiserum from these volunteers afforded significant protection of AKR mice challenged with cell-free filtrates of leukemic human brains. This protection was not afforded by pooled normal serum, and was apparent at various dilutions of the challenging material.

‡ Obtained from Michael Reese Research Foundation, Chicago, Ill.

§ Obtained from Jackson Memorial Laboratory, Bar Harbor, Me. or Millerton Farms, Millerton, N. J.

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Received December 4, 1959. P.S.E.B.M., 1960, v103.

Metabolism of Guanidoacetic Acid in Tumor-Bearing Rats.* (25544)

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We outlined(1) observations on some phases of creatine metabolism in rats bearing Walker carcinoma 256 and showed that there was a net decrease in creatine synthesis in these animals. Creatine synthesis may be considered in 2 steps: (a) formation of guanidoacetic acid (GAA) from arginine and glycine, and (b) methylation of GAA to give creatine. Obviously, an impairment in either one or both of these synthetic steps can result in reduction of creatine synthesis. Therefore formation and excretion of GAA in tumor-bearing rats were first studied to determine if rate of GAA production could be a limiting factor in creatine synthesis in these rats.

Materials and methods. Male rats of both Sprague-Dawley strain and Wistar strain (Carworth Farms) were used. No difference in data obtained with these 2 strains of animals was noticeable. Hence, results have been pooled and averaged. Animals weighing 150-300 g were paired by weight and fed a balanced semi-synthetic diet containing neither creatine nor GAA. One member of each pair received a subcutaneous transplantation of Walker carcinoma 256 and ate the diet *ad lib.*, the other member served as control and was pair-fed to its tumor-bearing mate. The animals were housed in individual metabolism cages to facilitate collection of urine samples. Food intake was recorded daily. Body weights were determined every fourth day and *in vivo* estimation of tumor weights(2) was made at same time interval. Urine collections were made for 2 successive days in each 4-day interval throughout the experiment. Thymol was used as a preservative. At various stages of tumor growth, animal pairs were sacrificed under ether anesthesia. Blood samples were withdrawn from the abdominal aorta and heparinized. Liver and kidneys were excised, weighed, and immediately frozen in liquid nitrogen. Frozen tissues were dried by freeze-dry technic. Dry tissues were weighed, ground to fine powder, and stored in deep freeze until analyses were made. Before analysis, the kidney powder was passed through a 60-mesh sieve, to give a more uniform product. The enzyme, transamidinase, which catalyzes the formation of GAA from arginine and glycine, was first found in the kidney(3). In our study, the activity of this enzyme was assayed as follows: To 25 mg of kidney powder were added 3 ml of M/15 phosphate buffer, pH 7.4, and 1.5 ml each of glycine (20 μmoles/ml) and of L-arginine (20 μmoles/ml). After thorough mixing, an aliquot of 2 ml of the suspension serving as control was taken, adjusted to pH 6, and kept in boiling water bath for 10 minutes. Upon cooling, the boiled tissue suspension was filtered. The protein-free filtrate was used for determination of GAA by chromatographic-colorimetric method(4). This method was used also for determination of GAA in plasma, tissues and urine. Another aliquot of 2 ml of kidney tissue suspension was pipetted into a small Erlenmeyer flask and incubated in Dubnoff shaking incubator at 37° for one hour. At end of incubation, the contents of flask were adjusted to pH 6 and subsequently treated in the same manner as the control sample. The difference in GAA values between control sample and incubated sample represents the amount of GAA synthesized under conditions employed. When liver was used in determination of GAA, the recovery of added GAA was not always satisfactory. The recovery was less with larger samples of liver. Presumably, this is due to presence in liver of certain substances interfering with the Sakaguchi reaction used for determination of GAA. However, if amount of liver used is small, quantitative recovery of added GAA can be obtained. It was satisfactory to use a protein-free filtrate prepared as for the kidney and equivalent to no more than 70 mg of dry liver for determination of GAA. Plasma

* This investigation was supported in part by research grant from Nat. Cancer Inst., U.S.P.H.S.

TABLE I. Kidney Transamidinase Activity of Tumor-Bearing Rats. All values expressed as μmoles of guanidoacetic acid synthesized/g of dry kidney/hr.

Type animal	Tumor size		
	Small	Medium	Large
Control	58.4 (48.7-65.1)† (3)*	52.3 ± 8.5‡ (6)	42.4 ± 5.4 (8)
Tumor-bearing	48.6 (30.6-61.0)	36.5 ± 14.6§	12.3 ± 2.8§

* No. of pairs of animals used. † Range. ‡ Value following ± sign is stand. error of mean. § Difference in mean values between control and exp. groups is significant by the "t" test.

was deproteinized with 4% trichloroacetic acid, final concentration, and the protein-free filtrate was used for the determination.

Results. For the purpose of correlating biochemical findings with different stages of tumor growth, an arbitrary classification based on tumor weight expressed as per cent of total body weight (carcass plus tumor) has been adopted. Animals with tumors weighing less than 10% of total body weight were grouped under "Small," those with tumors weighing between 10 and 30% were grouped under "Medium," and those with tumors weighing more than 30% were grouped under "Large."

Transamidinase activity of the kidney was lower in tumor-bearing rats than in controls (Table I). Furthermore, lowering in enzyme activity was more marked in rats with larger tumors. Thus, transamidinase activity in rats bearing large tumors was only one-fourth that in rats bearing small tumors. Although a depletion of protein intake resulted in reduction of this enzyme activity in the rat(5), and inanition usually develops in animals with large tumors, the use of the pair-feeding technic has ruled out possible implications of dietary restriction on our observations in tumor-bearing rats. Actually, the difference in enzyme activity between control animals pair-fed to animals bearing large tumors and those pair-fed to animals bearing small tumors, was not great, so that any effect of dietary restriction must have been slight. It may be concluded, therefore, that kidney transamidinase activity was decreased as a result of tumor growth.

A decrease in enzyme activity in a tissue measured *in vitro* does not necessarily mean a decrease in rate of a synthetic reaction catalyzed by the enzyme *in vivo*(6,7). This raised the question whether rate of production of GAA was really decreased in tumor-bearing animals which showed reduced transamidinase activity as studied *in vitro*. No direct evidence is available to answer this question. In an effort to gain information on *in vivo* production of GAA, a study of concentrations of this substance in plasma, kidney and liver and its excretion in urine was undertaken. Fig. 1 shows urinary excretion of GAA for a control and a tumor-bearing rat throughout the period of tumor growth. The data for this Figure were obtained from a single pair of animals, but they are typical of the 7 pairs of animals whose urinary pattern of GAA excretion has been studied. In none of these animals was there noted significant difference between amount of GAA excreted by the tumor-bearing and control rat. In sharp contrast to the large increase in urinary excretion of creatine observed in the pre-terminal stage of tumor growth(1), urinary excretion of GAA was unchanged at this time. In other words, there was creatinuria without a correspondingly high level of GAA excretion. This situation is similar to that observed with experimental avitaminosis E(8). Whether the principles underlying creatinuria in these 2 cases are the same remains to be seen.

Concentrations of GAA in plasma and kidney of tumor-bearing rats were the same as control values (Table II). Its concentration in liver, however, was increased in rats with

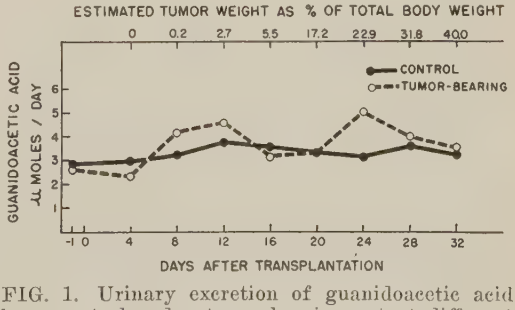

FIG. 1. Urinary excretion of guanidoacetic acid by a control and a tumor-bearing rat at different stages of tumor growth.

TABLE II. Guanidoacetic Acid in Plasma, Kidney and Liver of Tumor-Bearing Rats. Values expressed as μmoles of guanidoacetic acid/100 ml of plasma or /g of dry tissue.

			Liver Tumor size		
Type animal	Plasma*	Kidney*	Small	Medium	Large
Control	4.2 ±.4‡ (5)†	5.4 ±.9 (17)	1.34 ±.34 (5)	1.32 ±.20 (5)	1.38 ±.25 (5)
Tumor-bearing	4.3 ±.5	5.2 ±.7	1.67 ±.32§	1.74 ±.23§	2.09 ±.42§

* Values in plasma and kidney were obtained from animals with medium and large tumors, and averaged.

† No. of pairs of animals used.

‡ Value following ± sign is stand. error of mean.

§ Difference in mean values between control and exp. groups is significant by the "t" test.

tumors. The increase appears to be greater in animals with larger tumors. This indicates that ability of liver to concentrate GAA was not impaired by tumor growth. The fact that concentration of GAA actually was increased in the liver of tumor-bearing animals suggests that there was a decrease in utilization of GAA for creatine synthesis. This suggestion is consistent with our previous observation of decreased creatine synthesis in these animals (1). Since concentrations of GAA in plasma and kidney and its excretion in urine were unchanged, and its concentration in liver was somewhat elevated in tumor-bearing rats, it appears probable that the synthesis of GAA proceeded at a rate comparable to that in control animals. However, the possibility that rate of GAA synthesis could have been reduced to be commensurate with decreased creatine synthesis and still result in the observed levels of GAA in tissues, plasma and urine, cannot be excluded. This possibility will be tested further.

Summary. Transamidinase activity in the kidney of rats bearing Walker carcinoma 256 was lower than that of the control. However, concentrations of guanidoacetic acid in plasma and kidney and its excretion in urine of tumor-bearing rats were not changed. In the liver, concentration of this compound was increased with tumor growth. These findings seem to indicate that rate of guanidoacetic acid synthesis is not a limiting factor in synthesis of creatine by the tumor-bearing rat.

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Received December 7, 1959. P.S.E.B.M., 1960, v103.

Kaolin Treatment of Sera for Removal of Nonspecific Inhibitors to Asian Strains of Influenza Virus.* (25545)

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Kaolin is used for removal of nonspecific serum inhibitors to the arthropod-borne viruses(1). It has also been used to remove nonspecific inhibitors to Asian influenza viruses from human and fowl sera and there is some evidence that treatment of sera with kaolin permits more cases of Asian influenza to be detected by the hemagglutination-inhibition (HI) test than treatment with trypsin (2). Kaolin has advantages over other substances used routinely for removal of nonspecific serum inhibitors to the influenza viruses, *viz.* trypsin, periodate and receptor destroying enzyme (RDE), in that it is easily available and relatively cheap. Furthermore, it does not have to be prepared fresh before use and treatment of a serum with kaolin can be accomplished within an hour, treatment overnight being unnecessary. The present study was undertaken to investigate the value of kaolin in routine diagnostic work with the Asian influenza viruses and to compare kaolin treatment with other technics.

Materials and methods. The HI test was carried out in the manner described by Jensen (3) with one modification, phosphate buffered saline (pH 7.2) replacing normal saline as diluent. Complement-fixation (CF) test was done by the method of Casals(4). Antigens were infected allantoic fluids. Sera were treated with periodate in the usual manner, trypsin(3), RDE(5) and kaolin and acetone (1). Treatment with kaolin(1) consists essentially in the following: to 1 volume of serum are added 4 volumes of borate-saline buffer at pH 9.0 and 5 volumes of a 25% suspension of kaolin† in borate-saline buffer. The mixture is held at room temperature for 20 minutes, centrifuged and the supernatant used.

Results. Acute and convalescent sera from 9 cases of Asian influenza, diagnosed by virus isolation during an epidemic in Trinidad in 1957, were treated by 3 different methods and tested by HI with the Asian strain A/Asian/Japan/305/57. Results are shown in Table I, which also gives the results of CF tests on the same sera. Kaolin treatment permitted 7 of the 9 proven cases of influenza to be diagnosed. Six cases each were diagnosed by the use of periodate and RDE.

In addition, sera from other cases diagnosed clinically as influenza but not confirmed by virus isolation were tested under the same conditions. Table II summarizes the results on all the sera tested, including those shown in Table I. When all the cases are considered, the results with kaolin compare favorably with the other methods.

The effectiveness of various technics in removing from normal fowl serum nonspecific inhibitors to 2 Asian influenza strains isolated in Trinidad is shown in Table III. Two other strains, B/GL/1739/54 and A/Denver/1/57, are included as controls. With normal fowl serum the best results were obtained with trypsin. Kaolin removed most of the inhibitor and gave better results than all of the other methods except trypsin.

Discussion. The methods used for removal of nonspecific inhibitors to influenza virus are not entirely satisfactory. In this laboratory trypsin treatment has not given reproducible results with the Asian strains. With other non-Asian strains treatment with trypsin has often been found to be disappointing, and there is no assurance that any of the treatments do not reduce specific antibody levels(6). Many of the Asian influenza isolates are very sensitive to nonspecific inhibitors in heated sera and treatment of sera with trypsin, periodate or *Vibrio cholerae* extracts

* Studies and observations were conducted by the Trinidad Regional Virus Laboratory with support and under auspices of Government of Trinidad and Tobago, the Colonial Development and Welfare Scheme and The Rockefeller Foundation.

† Obtained from Amend and Co., New York, N. Y. Specify acid-washed product.

TABLE I. Comparison of 3 Methods of Removing Nonspecific Inhibitors from Sera of Proven Cases of Asian Influenza.

	CF titers		HI titers					
			Sera treated with					
			Kaolin		Periodate		RDE*	
Antigen	Acute	Conv.	Acute	Conv.	Acute	Conv.	Acute	Conv.
A/Asian/Japan/305/57	8	128	10	80†	10	0	10	0
	0	64	0	20	10	20	0	40
	8	32	10	160	0	20	0	160
	0	8	10	40	0	20	0	10
	0	16	10	40	0	40	0	40
	0	32	0	20	0	20	0	20
	0	32	10	160	0	80	0	40
	8	128	20	40	0	40	0	20
	0	8	10	10	0	0	0	0

* Receptor destroying enzyme.

† Fourfold (= 2 tube) or higher rise in titer from acute to convalescent specimen accepted as evidence of infection.

TABLE II. Number of Cases of Influenza Diagnosed by the Hemagglutination-Inhibition Test When 3 Different Methods of Serum Treatment Were Employed.

Method of treatment	No. of cases tested	No. positive	% positive
Periodate	29	18	62
Kaolin	31	19	61
RDE	19	8	42

does not remove these troublesome inhibitors (7). As it is likely that the Asian strains will be prevalent for a number of years, alternative methods of removing inhibitors will be needed. These preliminary investigations suggest that kaolin may be useful in this respect. The possible use of kaolin for removal of nonspecific inhibitors to other myxoviruses should also be considered.

TABLE III. Treatment of Normal Fowl Serum by Various Methods for Removal of Nonspecific Inhibitors to Asian Strains of Influenza Virus Isolated in Trinidad.

	Virus strains			
Method of treatment of fowl serum	B/GL/ 1739/54	A/Denver /1/57	Trinidad 17359	Asian 17337
No treatment	0	0	40*	80
Inactivation at 56°C/30 min.	0	0	640+	640+
Kaolin	0	0	10	10
Kaolin + inactivation at 56°C /30 min.	0	0	20	20
Trypsin	0	0	0	0
Periodate	0	0	80	160
RDE	0	0	160	320
Acetone	0	0	160	80

* Reciprocal of maximum serum dilution which inhibited hemagglutination by 4 units of antigen.

During investigations on another outbreak of influenza in the summer of 1959 in Trinidad, the value of kaolin was again demonstrated. Six viruses were identified as Asian strains of influenza virus by the use of HI tests with kaolin treated fowl antisera. Periodate treatment failed to remove the inhibitor from the same antisera. Eleven cases diagnosed by the CF test and/or virus isolation were also diagnosed by the HI test using kaolin treated sera.

In view of the advantages of kaolin treatment over other technics further trials of this method are clearly indicated.

Summary. The use of kaolin for treatment of sera in diagnosis of Asian influenza cases by hemagglutination-inhibition test is reported. Comparison of kaolin treatment with other methods in common use is made. The advantages of kaolin treatment over other technics warrant further trial of the method.

The receptor destroying enzyme was kindly furnished by Dr. Frank L. Horsfall, Jr., Rockefeller Institute, N. Y.

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Received December 7, 1959. P.S.E.B.M., 1960, v103.

Effect of Ethionine on Amino Acid Composition of Rat Tumors.* (25546)

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We have shown that ethionine inhibited growth of 4 different tumors in rats but its effectiveness varied with strain and sex of host animals(4,12,14,18). Inhibition by ethionine of growth of tumors in mice has been reported from other laboratories(6,23, 26). Although many studies have been made (7,8,11,16,19,24,25,28-32), it is not clear whether ethionine inhibits incorporation of methionine *per se,* or of other amino acids, into proteins of the tumor or causes decreased growth of tumors by affecting adversely protein synthesis or other metabolic processes elsewhere in the body. It was reported(4,5, 12-15,18) that concentration of some "free" amino acids is decreased, and some increased, in liver of rats treated with ethionine. Our object was to determine whether composition of proteins, or concentration of "free" amino acids, in tumors of rats is affected by administration of ethionine.

Materials and methods. Young male and female rats (average weight 174 and 162 g, respectively) of Wistar strain, maintained on *ad lib.* diet of Purina Chow supplemented with weekly ration of fresh carrots, were divided into groups and treated as shown in Table I. Eighteen to 24 hours after last injection, the tumors were removed under ether anesthesia, freed as much as possible from connective tissue, rinsed with distilled water, blotted and weighed. The method of sample preparation was based on that of Schurr *et al.*(22). The viable tissue of each tumor was separated quickly from the necrotic portion, rinsed, and heated for 3 minutes in boiling water. The boiled viable tissues of each group were blotted free of water, separated from small amount of necrotic tissue remaining, cut into small pieces and pooled. The pooled samples were homogenized in Waring Blender and concentrated *in vacuo* to an appropriate volume depending on size of group sample. These suspensions were analyzed for dry weight, total nitrogen(9,27), total amino acids, and apparent free amino acids. Ash and mineral data obtained on these samples will be reported elsewhere. *Total Amino Acids*: Aliquots of tissue suspensions were refluxed in 8 N HCl for 24 hours and amino acids determined by microbiological assay methods previously described(2,3,14). *Apparent Free Amino Acids*: Aliquots of tissue suspensions were deproteinized with tungstic acid(22) and the amino acids determined by microbiological assay procedures described previously(2, 4,14).

Results. Data on number and sex of animals, tumors inoculated and material injected are given in Table I. Dosage of ethionine in females was reduced to ½ that used for males, to obtain a comparable degree of tumor inhibition in both sexes, since Dunn *et al.*(5,18) and Simpson *et al.*(25) reported that ethionine was more toxic, a more potent tumor-growth inhibitor, and affected liver composition to greater degree in female than in male rats.

Seven injections of ethionine inhibited growth of Sarcoma R-1 55% and 40%, and Walker carcinosarcoma 256 74% and 39% in males and females, (data omitted to conserve space). Degree of tumor inhibition ob-

* Paper No. 130. This work was supported by grants from Am. Cancer Soc., U.S.P.H.S. and Univ. of California.

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TABLE I. Experimental Design.

Animals* No./group	Sex	Tumor inoculated†	Material inj. Type	Material inj. Daily amt‡
15	♂	Sarcoma R-1	Saline§	10 ml
20		*Idem*	Ethionine‖	100 mg/100 g body wt
23		Walker carcinosarcoma 256	Saline	10 ml
30		*Idem*	Ethionine	100 mg/100 g body wt
20	♀	Sarcoma R-1	Saline	5 ml
39		*Idem*	Ethionine	50 mg/100 g body wt
24		Walker carcinosarcoma 256	Saline	5 ml
35		*Idem*	Ethionine	50 mg/100 g body wt

* Range of avg weights of each group was as follows: males, 173-174 g; females, 158-164 g.
† A cube, approximately 85 mm^3, was implanted subcut. by trocar technic into 2 axiliary sites of each animal.
‡ From 5th to 8th, and 11th to 13th day, inclusive, following tumor implantation.
§ 0.85% aqueous solution of sodium chloride.
‖ 2% solution of DL-ethionine in 0.85% aqueous solution of sodium chloride.

served in present experiments was about the same for females, but was greater for males, compared to results obtained previously(18). Growth of both tumors in male rats was less than expected, the decreased tumor growth and poor condition of treated males necessitating a 2-day interruption of injections (Table I). There is no simple explanation for this slower tumor growth but apparently it was not due to decreased viability of tumors *per se,* since normal growth was observed only a month before and subsequent to present experiment. As animals were purchased from commercial source, it is possible that some variation in host animals was responsible for this result.

Total Amino Acids. Percentages (dry-weight basis) of total amino acids in viable tissue of the 2 tumors are given in Table II. There was no significant difference in amino acid composition between the 2 tumors grown in male or female, treated or untreated rats whether values were calculated on dry-weight basis or on nitrogen content (data omitted to conserve space) of samples. Close agreement (average difference, 3.5%) between duplicate values was observed for aspartic acid, glutamic acid, histidine, isoleucine, leucine, lysine, proline, serine and threonine. Only single assay values were obtained for arginine, glycine, methionine, valine and the indicated samples of the other amino acids.

There were no significant differences between percentages of 13 amino acids found for the 2 rat tumors studied, the UCLA rat fibrosarcoma(3) and the 4 rat tumors investigated by Sauberlich and Baumann(21). It is of interest, therefore, to note the marked differences in aspartic acid, lysine and serine for these rat tumors compared to mouse tumor (Sarcoma 180) reported by Mickelson and Barvick(17). The problem of possible species difference in this regard is under investigation.

Apparent Free Amino Acids. μg/100 mg (dry-weight basis) of apparent free amino acids in viable tissue of the 2 tumors are given in Table III. The average difference between analyses made in duplicate was relatively high (11%) owing to difficulties encountered in handling and assaying the small quantities of available material. Levels of threonine in tumors were consistently higher in female than in male rats (Table III). Injection of ethionine had no effect on level of methionine in the tumors; the one inconsistent value (Sarcoma-males) is believed not to be significant.

Levels of arginine and histidine in viable tissue of the Walker tumor and of lysine in both tumors were lower in experimental animals of both sexes. It is of interest that "free" arginine and lysine (histidine was not determined) were elevated in liver after treatment with ethionine(4,5) while generally they were decreased in tumors in present experiments. It is of further interest that Busch and Davis(1) reported that specific activity of histones of Walker and Jensen tumors as measured by uptake of L-lysine-U-C^{14} or of glycine-1-C^{14}, was greater than those of pro-

TABLE II. Total Amino Acid Concentration of Viable* Tissue of Sarcoma R-1 and Walker Carcinosarcoma 256 Tumors Grown in Control and Ethionine-Treated Male and Female Rats of Wistar Strain. Values expressed in % of dry wt of original tissue.

	Sarcoma R-1				Walker carcinosarcoma 256			
	♂		♀		♂		♀	
Amino acids	Control	Exp.	Control	Exp.	Control	Exp.	Control	Exp.
Arginine	5.0 (.71)†	5.4 (1.1)	5.0 (.89)	5.1 (1.7)	5.1 (2.6)	5.0 (2.4)	5.3 (2.0)	5.7 (1.6)
Aspartic acid	6.8 (.25)	6.9‡ (3.4)	7.0‡ (2.0)§	6.6 (2.8)	7.2‡ (2.0)	7.3‡ (4.5)	7.1‡ (.82)	7.2‡ (4.6)
Glutamic acid	9.6 (8.9)	9.6‡ (9.5)	10.0‡ (10)§	10.2‡ (8.3)	9.6 (7.3)	9.5‡ (4.0)	10.0 (8.8)	9.8 (5.9)
Glycine	4.3 (1.6)	4.5 (1.2)	4.3 (2.5)	4.6 (.34)	4.7 (.68)	4.5 (2.3)	4.3 (.14)	4.7 (3.0)
Histidine	1.8 (3.4)	1.9 (4.8)	1.8‖	1.9‡ (3.4)	2.0‡ (3.6)	1.9 (2.6)	2.0‡ (2.4)	2.1‡ (2.4)
Isoleucine	3.5 (1.8)	3.7 (.33)	3.4‡ (1.5)§	3.3‡ (4.2)	3.6‡ (.53)§	3.5‡ (1.0)	3.6‡ (.87)§	3.8‡ (2.4)
Leucine	6.3 (.66)	6.6 (2.8)	6.1 (2.5)	6.0 (.87)	6.4‡ (3.2)	6.4‡ (1.6)	6.4‡ (.88)	6.5‡ (1.4)
Lysine	7.0‡ (3.6)	6.6 (2.9)	7.1‡ (1.7)§	7.2‡ (4.2)	7.2‡ (4.1)	7.0‡ (1.8)	7.1‡ (2.0)	7.8‡ (4.2)
Methionine	1.6 (3.7)	2.0 (7.1)	1.7 (1.8)	1.6 (4.7)	1.8 (3.5)	1.8‖	1.7 (4.2)	1.8 (4.3)
Proline	3.6 (3.3)	3.7‡ (3.5)	3.7 (1.8)	3.7 (2.2)	3.6 (3.2)	3.7 (1.5)	3.7 (2.1)	3.6 (4.0)
Serine	3.8‡ (4.2)	3.8‡ (6.1)	3.7 (3.1)	3.6 (3.8)	3.8 (5.5)	4.2‡ (6.5)	4.0‡ (4.9)	4.1 (3.2)
Threonine	3.7‡ (.59)§	3.6‡ (2.4)	3.6‖	3.6‡ (2.7)§	3.4 (1.7)	3.6‡ (1.6)§	3.4‡ (2.8)	3.6‡ (1.4)
Valine	4.3 (.67)	4.6 (1.1)	4.2‖	4.4 (1.2)	4.6 (.67)	4.3 (.36)	4.4 (.13)	4.6 (1.4)

* Comprised approximately 85% of Sarcoma R-1 and 55% of Walker carcinosarcoma 256 on dry wt basis.
† Mean deviation from mean, MDM, in %, over a 3- to 5-fold range of concentration of arginine and all other assay samples.
‡ Avg of duplicates, all others single values. Mean of deviations among duplicates, 3.48%.
§ MDM of second determination; in original assay only 2 levels fell on standard curve.
‖ MDM not calculated since only 2 values for levels of samples fell within standard curve; assay not repeated owing to insufficient amount of sample.

TABLE III. Apparent Free Amino Acid Concentration of Viable Tissue of Sarcoma R-1 and Walker Carcinosarcoma 256 Grown in Control and Ethionine-Treated Male and Female Wistar Rats. Values expressed as μg/100 mg dry wt of tissue.

	Sarcoma R-1				Walker carcinosarcoma 256			
	♂		♀		♂		♀	
Amino acids	Control	Exp.	Control	Exp.	Control	Exp.	Control	Exp.
Arginine	12 (6.5)*	9 (3.4)	11 (4.7)	11 (3.5)	21 (4.1)	13 (1.2)	19 (4.1)	13 (2.6)
Aspartic acid	20†	26†	40§ (4.4)‡	37§ (4.2)‡	35 (2.2)‡		27 (3.7)	27 (4.7)
Glutamic acid	113 (35)‡	138 (23)	197§ (4.5)‡	178§ (11)‡	123§(4.5)‡	107 (6.2)‡	105 (3.2)‡	132§(4.0)‡
Glycine	79 (1.6)	93 (1.7)	97 (2.4)	108 (2.7)	97 (3.0)	103 (2.5)	105 (2.1)	109 (3.2)
Histidine	4†		4 (8.4)‡	6 (4.5)‡	7 (4.5)	3 (5.3)‡	8 (2.7)‡	5 (1.4)
Isoleucine	10 (27)‡	14 (4.9)	16 (2.8)	14 (1.0)	16 (.46)	7 (7.7)	13§(5.2)	12 (8.8)
Leucine	20†	26†	28 (4.9)	25 (2.2)	32 (4.7)	17 (10)	20 (3.7)	18 (2.3)
Lysine	31 (5.6)	25 (3.6)	40 (.78)	34 (.50)	52 (4.8)	19 (5.2)	37 (2.2)	29 (5.3)
Methionine	16 (11)‖	25 (5.5)‖	14 (4.4)‖	13 (7.4)‖	15 (2.2)‖	15 (11)‖	11 (5.5)‖	11 (7.9)‖
Proline	43 (19)‡	45 (8.5)‡	73 (4.5)‡	52§ (5.2)‡	33 (7.5)‡	34 (6.4)‡	36 (6.6)‡	37 (.96)
Serine	35 (3.3)‖	45 (6.3)‖	31 (13)‖	53§ (7.5)‖	47§(6.1)‖	45 (7.0)‖	47§(7.8)‖	56 (6.9)‖
Threonine	38 (9.8)‡	36 (8.0)‡	56 (5.9)‡	60 (3.3)‡	36 (2.6)	37 (1.5)	40 (3.1)	72 (.90)
Valine	17 (23)‡	19 (21)‡	36 (6.5)‡	22§ (6.9)‡	27 (3.6)‡	13 (11)‡	18 (2.5)‡	17 (3.9)‡

* Mean deviation from mean, in %, over a 3- to 5-fold range of concentration of arginine and all other assay samples (MDM).
† Fifth level of sample concentration only.
‡ Drift (values *increased* from 1st toward 5th level of sample concentration).
§ Analysis in duplicate.
‖ Drift (values *decreased* from 1st toward 5th level of sample concentration).

teins of any other cellular fraction of the tumor, and that specific activity of histones and other nuclear proteins of these tumors was greater than that of 11 other tissues. It is possible that interference with metabolism of one or more of the basic amino acids in liver following ethionine injection could contribute to "starvation" of tumor cells.

Lack of consistency in differences observable in isoleucine, leucine, proline, and valine is believed to render these differences insignificant.

No analogous microbiological assay data are available but the present values for aspartic acid, glutamic acid, glycine, and valine are within, while that for histidine is below, the range of values reported by Kit and Awapara(10) and Sassenrath *et al.*(20) who used chromatographic technics. It is possible that some free amino acids originally present in viable tissue were removed by boiling. Since relative (not absolute) values are being sought here this should not be a major objection to the method.

Summary. 1. Percentages of total and "free" amino acids in viable tissues of Sarcoma R-1 and Walker carcinosarcoma 256 grown in control and ethionine-treated male and female rats of the Wistar strain have been determined by microbiological assay methods. 2. Ethionine treatment induced no change in total amino acids but caused an increase or decrease in concentration of some "free" amino acids in viable tissue but was without marked effect on methionine. 3. It was concluded that inhibition of tumor growth by ethionine probably results largely from indirect effects on utilization of amino acids other than methionine.

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Received December 8, 1959. P.S.E.B.M., 1960, v103.

Modification of Lysozyme Thermostability by Cytochrome c Preparations.* (25547)

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In fractionating rat kidney lysozyme by ion-exchange chromatography, most active fractions contained high concentrations of Cytochrome c(1) subsequently resolved after rechromatography(2). Because of similarities in physico-chemical properties, it was considered that Cytochrome c might affect or imitate the functions of lysozyme in some way. Many basic polypeptides and small peptides exert bacteriolytic activity(3-5). Antibacterial activity has been attributed to hemoglobin and related compounds, including Cytochrome c, which affects oxidizing capacity of *Bacillus subtilis*(6). Kammerer(7) and van Heyningen(8) claimed that mesohematin and hematin, respectively, inhibited bacteria. This report describes 2 effects of Cytochrome c: (a) growth of *Micrococcus lysodeikticus,* frequently used as a substrate for lysozyme, and (b) *in vitro* activity and thermostability of lysozyme.

Materials and methods. Crystalline egg white lysozyme was obtained from Armour Research Labs., Chicago. Two preparations of Cytochrome c were used: beef heart Cytochrome c (Sigma Chem. Co., St. Louis) and horse heart Cytochrome c ("Wyeth Injection Cytochrome c," Philadelphia, 10 mg/ml). The redox state of Cytochrome c was determined by measurement of absorption spectrum in the visible range. Reduction of Cytochrome c was accomplished by excess of $Na_2S_2O_4$. *M. lysodeikticus* (strain 19, Rutgers Inst. of Microbiology) was cultured in liquid media at 28°C and growth followed photometrically at 660 mμ by previously described procedure(9). Cytochrome c or lysozyme was dissolved in sterile water and passed through bacterial filter before addition to culture medium. *M. lysodeikticus* cells used for lysozyme substrate were prepared and standardized by published method(10,11). Lysozyme activity was measured by reduction in turbidity of a bacterial suspension(10) and may be defined as change in percent transmission at 645 mμ during 30 to 60 seconds of reaction in standard assay system (1 unit = change of 1% transmission in this interval) (10). Enzyme activity measurements were replicated to within ± 0.2 units. Following incubation of lysozyme in presence or absence of Cytochrome c at various temperatures for various periods, samples were transferred to ice-water bath, pH was measured, and 0.25 ml aliquot assayed in standard assay system buffered at pH 6.2(10). Heat treatments were performed on aqueous solutions or on solutions buffered by either 0.1 *M* acetate at pH 5.0, or 0.066 *M* phosphate at pH 7.0.

Results. Effect of Cytochrome c upon growth of M. lysodeikticus: A mutational lag phase occurred after lysozyme was added to cultures of *M. lysodeikticus*. Eventually, resistant organisms developed(9). Inclusion of levels of Cytochrome c in cultures of the organism inhibited growth, without a lag, in contrast to results of lysozyme action. Concentrations of Cytochrome c in the culture varied from 0.1 to 0.4 μg/ml. Inhibition of bacterial growth at these levels and extrapolation of Cytochrome c concentration as a function of growth rate (logarithmic phase) revealed that 100% inhibition of growth occurred at Cytochrome c concentration of about 1.1 μg/ml of culture medium. Similar results were obtained if Cytochrome c concentration was expressed as a function of total cellular Kjeldahl nitrogen.

Thermostabilization of lysozyme activity in presence of Cytochrome c: 100 μg of lysozyme were combined with various concentrations of Cytochrome c in an aqueous, unbuffered solution (total volume = 4 ml) and subjected to 100°C water bath for 10 minutes. Results obtained are shown in Fig. 1. The control curve containing oxidized Cytochrome c in-

* Supported in part by research grant, from Nat. Science Fn. Journal Series, N. J. Agr. Exp. Station.

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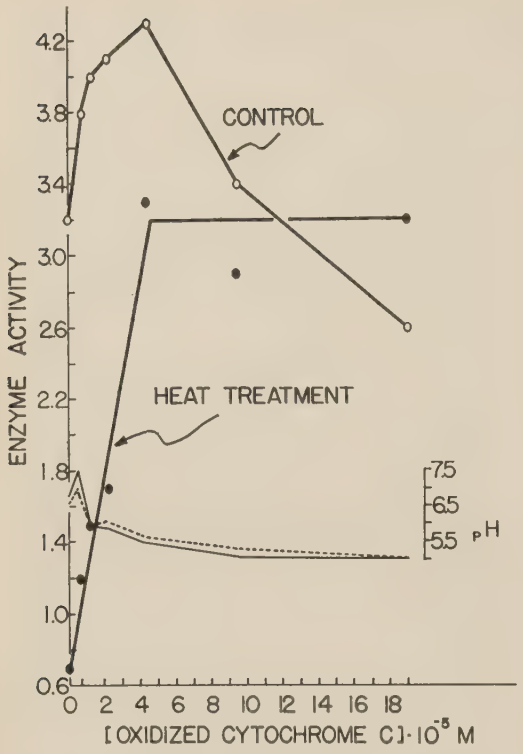

FIG. 1. Thermostabilization of lysozyme activity in aqueous solution by Cytochrome c. Control curve represents enzyme activity resulting from preparations not treated by heat. Heat treatment, 10 min. at 100°C with varying concentrations of oxidized Cytochrome c. pH curves reveal values of systems (after incubation) prior to enzyme assay. Solid curve = control; dashed line = heat treatment. See text for further details of aqueous system.

dicates a stimulation in initial lytic activity over a given concentration range of the pigment. Reduced Cytochrome c also caused a slight stimulation in initial lysozyme activity but was inactive in stabilizing lysozyme to heat. The reasons for inability of the reduced pigment to affect thermostabilization of lysozyme are not clear at this time. The reducing agent, $Na_2S_2O_4$, alone had negligible activity in bacteriolysis. Either oxidized or reduced forms of the pigment failed to produce measurable *in vitro* lysis in absence of lysozyme. The curve of results of heat treatment shows that in similar concentration range of oxidized Cytochrome c, stability of lysozyme activity to heat is increased. The optimal level of Cytochrome c for stimulation or thermostability is about $5 \cdot 10^{-5}$ *M*, assuming molecular weight of 13,000(12); at this point the ratio of Cytochrome c molecules to lysozyme molecules is about 10 to 1. The resulting pH values of the systems, with or without heat treatment, rose and then gradually fell to pH about 5.1. The pH after heat treatment was not greatly different from the unheated control.

Systems containing ratios of about one lysozyme molecule to about 13 molecules of Cytochrome c were utilized to study the thermostabilizing effect of the pigment as a function of temperature and pH following exposure periods of 0 to 90 minutes. Significant protection was afforded by Cytochrome c in incubates buffered at pH 5.0 at 28°C, 37°C and 60°C. Compared to zero time control an element of stimulation appeared combined with the thermostabilizing effect. Results at pH 7 were much less dramatic than systems exposed to heat at pH 5.0.

Discussion. Stimulation of initial lysozyme activity by Cytochrome c may result from increased number of $-NH_3^+$ groups provided in the Cytochrome peptide. Ionized amino groups are essential for lysozyme activity(13). It seems possible that some active sites of lysozyme molecules, involving $-NH_3^+$ groups could be adsorbed to inert sites on the bacterial cell wall. If Cytochrome c also could be adsorbed to inert positions on the cell wall, the total concentration of net effective lysozyme active sites might be increased.

Thermostabilization of lysozyme may result because of similar physico-chemical properties which might confer to Cytochrome c a "buffering" protection effect upon the active site of lysozyme. These ideas represent pure speculation.

The inhibitory effect of Cytochrome c upon bacterial growth and the thermostabilizing or stimulating action of this substance may not necessarily involve the same portions of the cytochrome molecule (*viz.* growth inhibitory effect may reside in the heme portion; stimulation or stabilization may involve the peptidic structure).

Summary. Preparations of Cytochrome c from horse heart or beef heart were potent inhibitors of growth of *Micrococcus lysodeikticus*. Preparations of oxidized Cytochrome c from either beef heart or horse heart increased stability of lysozyme activity to heat under

certain conditions. Cytochrome c reduced by $Na_2S_2O_4$ does not appear to stabilize lysozyme activity to heat. Protection of lysozyme activity by oxidized Cytochrome c is afforded from 28°C to 60°C in solutions buffered at pH 5.0 to 90 minutes. Excellent protection is afforded also to aqueous, unbuffered solutions at 100°C for incubation of 10 minutes. The optimal molecular ratio for exertion of beneficial effects upon lysozyme appears to be about 10 molecules of Cytochrome c to 1 of lysozyme. Under certain conditions Cytochrome c preparations appear to exert a slight stimulation of initial velocity of lysis by lysozyme.

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Received December 11, 1959. P.S.E.B.M., 1960, v103.

Effect of Beta-Aminopropionitrile and Cholesterol on Lipids and Aortic S^{35}-Sulphated Mucopolysaccharides in Cockerels. (25548)

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When fed to animals and birds, Lathyrus factor beta-aminopropionitrile (BAPN) results in severe osteoporosis, skeletal deformities, dissecting aneurysms of aorta and various internal hemorrhages(1,2). The toxic action of BAPN is related to defective metabolism of connective tissue and lesions in lathyrism are due to abnormal formation or destruction of mucopolysaccharides of ground substance(3,4). There seems to exist an analogy between vascular lesion of connective tissue noted in lathyrism and some changes in vascular ground substance observed in animals with experimental atherosclerosis. Both histological and biochemical data seem to indicate, that alteration in mucopolysaccharides of connective tissue is involved in entrance of lipids into the arterial wall(6,7,8,9). Because of possible analogy between vascular lesions in experimental atherosclerosis and those noted in lathyrism, we studied the effect of BAPN and cholesterol, added separately or in combination to cockerels' diet, on some lipids and mucopolysaccharides of the aorta.

Materials and methods. Cockerels of White-Rock Broiler strain used for this study were obtained from a commercial hatchery when 8 days old and kept, in groups of 8 to 10, in thermostatic brooders. Experimental diets were supplied from 16 days of age. The BAPN-containing diet was prepared by drying a 1% aqueous solution of synthetic beta-aminopropionitrile fumarate (Abbott Laboratories) on a weighed amount of commercial chicken starter. The concentration of 0.025% of BAPN in the diet was used. The cholesterol diet contained 2% of cholesterol and 5% of olive oil. The following 4 diets were given to 4 groups of birds during a 4 week period: 1. Basal diet (normal control); 2. BAPN diet; 3. BAPN + cholesterol diet; 4. Cholesterol diet. Isotope was administered 24 hours before birds were killed. Radiosulphate (S^{35} in H_2SO_4, Atomic Energy, Canada) was injected subcutaneously in dosage of 1 mc/kg of bird's body weight. The

TABLE I. Average Weight of Body and Organs (in g) in Cockerels Fed Various Diets during 4 Weeks.

	Control diet	BAPN diet	BAPN + cholesterol diet	Cholesterol diet
No. of birds	26	21	19	28
Initial B.W.	166	178	157	175
Final "	950	826	768	804
Liver	22	21	26	27
Aorta	.33	.34	.31	.31
Femur	4.94	4.85	4.79	4.56

dose was dissolved in 5 ml of distilled water together with 40 mg of sodium sulphate (10, 11). The cockerels were anaesthetized, killed by decapitation and the blood collected. Serum and livers were used for determination of lipids. From each bird, aorta and femur were carefully dissected. Bone was studied to compare S^{35} uptake of the aorta with that of a tissue (bone) having specifically high sulphate-fixing capacity(10,11). Majority of aortas were studied for S^{35}-radioactive mucopolysaccharides following the method described previously(12,13). The specificity of S^{35} uptake method for study of certain mucopolysaccharides in tissue is well known. In some aortas lipid content was determined. The bones were dissolved by the nitric-perchloric acid wet-digestion procedure(14). The final precipitate of barium sulphate obtained after processing the tissues was transferred to planchettes and counting carried out using a thin mica window Tracerlab G-M tube. A Tracerlab Superscaler S.C. 18A was used for readings. Results were expressed in counts per minute (c.p.m.). Total cholesterol was determined following the method of Abell *et al.*(15). For study of lipid phosphate the samples were extracted with alcohol-ether and analysis carried out by the method of Zilversmit and Davis(16).

Results. All cockerels used in this experiment showed hock deformities and crooked toes of varying degrees of severity(2,11). Of cockerels receiving BAPN diet alone or with cholesterol about 16% died of hemorrhage due to aortic rupture.

Average weight of birds and organs used for biochemical studies is given in Table I. The results of study of lipids in serum, liver and aortas are given in Tables II and III. Cholesterol feeding produced a rise of total cholesterol in all birds. Addition of BAPN to the diet did not result in a marked alteration of lipid pattern in tissues studied. Table IV gives results of measurement of S^{35} uptake by the fraction of aortic tissue considered to contain sulphated mucopolysaccharides. Total radioactivity of femora of birds is also recorded. Increased S^{35} uptake by the sulphated mucopolysaccharides of the aortic wall in cholesterol fed cockerels was observed, and this finding agrees with our previous work (7). Cholesterol diet did not affect radioactivity of bones. Addition of BAPN to control diet or to atherogenic diet did not influence S^{35} uptake by aortas and bones.

Despite the fact that both BAPN and cho-

TABLE II. Serum and Liver Lipids in Cockerels Fed Various Diets during 4 Weeks.

	Control diet	BAPN diet	BAPN + cholesterol diet	Cholesterol diet
No. of birds	26	21	19	28
Serum total cholesterol, mg/100 ml	182 ± 17* (87-235)†	144 ± 11 (101-216)	617 ± 98 (311-1252)	1156 ± 161 (380-2090)
Serum phospholipids, mg/100 ml	271 ± 31 (193-363)	300 ± 17 (200-326)	392 ± 22 (395-520)	423 ± 25 (368-763)
Serum C/P‡	.51 ±.07 (.35-.74)	.42 ±.04 (.26-.58)	1.98 ±.33 (1.09-4.11)	2.81 ±.32 (1.10-3.81)
Liver total cholesterol, mg/100 g	367 ± 11 (324-423)	393 ± 11 (339-479)	4942 ± 136 (2877-7630)	6362 ± 466 (2759-8657)
Liver phospholipids, mg/100 g	2698 ± 72 (2373-3100)	2804 ± 82 (2075-3275)	2942 ± 76 (2450-3200)	2792 ± 89 (1850-3150)
Liver C/P	.14 ±.01 (.11-.18)	.14 ±.006 (.11-.17)	1.70 ±.20 (.97-2.75)	2.36 ±.42 (1.03-4.68)

* Mean ± S.E. † Range. ‡ Total cholesterol/phospholipids ratio.

TABLE III. Lipids of Aorta in Cockerels Fed Various Diets during 4 Week Period.

	Control diet	BAPN diet	BAPN + cholesterol diet	Cholesterol diet
No. of birds	10	11	9	12
Total cholesterol, mg/100 g	194 ± 12* (93-247)†	205 ± 18 (117-233)	372 ± 23 (275-524)	414 ± 50 (264-930)
Phospholipids, mg/100 g	704 ± 48 (465-843)	822 ± 22 (595-1115)	945 ± 30 (765-1075)	877 ± 35 (680-1123)
C/P	.29 ±.02 (.13-.38)	.26 ±.02 (.17-.32)	.40 ±.03 (.30-.55)	.47 ±.04 (.30-.83)

* Mean ± S.E. † Range.

TABLE IV. Uptake of S^{35} by Bone and Radioactivity of S^{35}-Sulphated Mucopolysaccharides of Aortas (S.M.) in Cockerels Fed Various Diets during 4 Week Period.

	Control diet	BAPN diet	BAPN + cholesterol diet	Cholesterol diet
No. of bones	26	21	19	28
S^{35} in bone, cpm/100 mg dry wt	1157 ± 43* (823-1649)†	1150 ± 76 (563-1621)	1265 ± 59 (856-1553)	1185 ± 72 (417-1590)
No. of aortas	16	10	10	16
S^{35} in S.M. fraction of aorta, cpm/100 mg dry wt	723 ± 51 (409-1052)	763 ± 60 (457-1109)	1878 ± 101 (1294-2320)	1564 ± 72 (1180-2034)

* Mean ± S.E. † Range.

lesterol diet may influence collagen metabolism of the vascular wall in birds, there was no marked analogy in the effect of these 2 factors on some aspects of lipid and mucopolysaccharide metabolism, which has been studied under the described experimental conditions.

Summary. 1) Beta - aminopropionitrile (Lathyrus factor) alone, or combined with cholesterol was added to diet of cockerels for 4 weeks. Normal diet or cholesterol rich diet was fed to control birds. BAPN feeding resulted in deformities of extremities and frequent death from aortic rupture. These changes were not influenced by addition of cholesterol to the diet. 2) BAPN had no effect on lipid content in serum, liver or aorta. Alteration in lipid pattern was obtained only with cholesterol rich diet. 3) Parallel to increase of lipids in tissues there was a significant rise of S^{35} uptake by sulphated mucopolysaccharides of the aorta in cholesterol fed cockerels.

Technical assistance of V. Viil, A. Kangaloo and L. E. Long, is gratefully acknowledged. Abbott Lab. of Montreal, Canada kindly offered Beta-Aminopropionitrile Fumarate (BAPN) for this project.

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Received December 11, 1959. P.S.E.B.M., 1960, v103.

Oxygen Consumption of Whole Animal and Tissues in Temperature Acclimated Amphibians. (25549)

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It has been established that in many poikilotherms there is a difference in metabolism in members of a species from warm and cold environments. Animals obtained from the cold having a higher rate of oxygen consumption than those from a warmer environment, when both are measured at an intermediate temperature. It may be reasonable to assume that in the intact organism total body oxygen consumption is the sum of the rates of the various tissues. Therefore, a study of tissue respiratory metabolism from cold and warm acclimated animals of the same species should indicate the relative contribution of each tissue to changes of oxygen uptake noted in the intact organism. Freeman(1) found increased respiratory metabolism in brain brei but not in skeletal muscle of goldfish (*Carassius auratus*). A similar finding was reported by Suhrman(2). Roberts(3) working with muscle slices from the crab found increased metabolism in tissue from cold acclimated animals. Many such diverse reports on the compensation for temperature in metabolism of poikilotherms have been excellently reviewed by Bullock(4). The present report is concerned with acclimation of frogs (*Rana pipiens*) and newts (*Triturus viridescens*) with a study of selected frog tissues to learn which tissues most nearly parallel the whole animal response to the changed environmental temperature. It is hoped that such information will provide a tissue which can be analyzed more extensively as to the mechanism involved in the change of metabolism noted during acclimation.

Methods. Adult *Triturus viridescens* and *Rana pipiens,* obtained from field collectors in Tenn. and Wisc., respectively, were maintained at room temperature (19-21°C) for one week before beginning of an experiment. The frogs were divided into 2 groups and placed in constant temperature incubators, one group at 5°C and the other at 25°C. Triturus treated in a similar manner, were maintained at 15°C and 30°C. None of the animals were fed during these acclimation experiments. Total body oxygen consumption was measured in a constant volume respirometer using the Warburg technic. The flasks used were specially designed with a volume of about 100 ml and a protected side arm for the CO_2 absorbant. Animals were not anesthetized and the position of an animal was noted at beginning of a determination period. If it had changed position those readings were not used. No difference in oxygen consumption could be detected between animals in which the shaker was employed and in those determinations in which it was not used. Tissue metabolism was measured manometrically in the Warburg according to the method of Umbreit *et al.*(5). Slices of liver, cardiac muscle (ventricle), sartorious muscle and strips of intact stomach wall were removed from animals selected at random from a particular acclimated group. The slices were placed in the flasks with chilled Mine's solution buffered with phosphate at pH 7.4(6). An additional sample of each tissue was placed in a weighing bottle for drying at 100°C for determination of dry weight. A series of *Triturus viridescens* was thyroidectomized according to the method of Adams(7). Respiratory metabolism was studied periodically during the pre- and post-thyroidectomy stages. The athyroid condition was verified by histological sections after termination of experiments.

Results. Total body respiratory metabolism of frogs is presented in Fig. 1. The higher rate of oxygen consumption for the 5°C acclimated animals was highly significant ($p = < .001$) when determined at 10°C, significant when measured at 20°C ($p = < .03$), but at 30°C oxygen uptake of both groups was practically identical.

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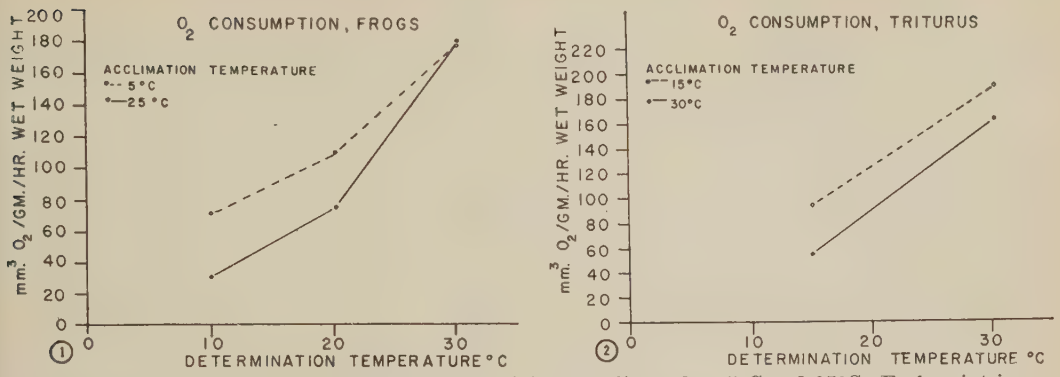

FIG. 1. Total body oxygen consumption of frogs acclimated at 5°C and 25°C. Each point is mean from 18 animals selected randomly from a population kept at specified temperature.

FIG. 2. Total body oxygen consumption of Triturus acclimated at 15°C and 30°C. Each point is mean from 12 determinations.

Triturus is also capable of making a metabolic adjustment in response to cold acclimation (Fig. 2); however, in Triturus there was a difference in the 2 acclimated groups ($p = < .01$) when O_2 uptake was measured at 30°C as well as at 15°C. In thyroidectomized Triturus whose respiratory metabolism was measured both before and after thyroidectomy, there was no difference in oxygen uptake between the 2 conditions. Some of these thyroidectomized animals were kept up to 60 days after operation. From the present experiments, it was concluded that the thyroid is not a major factor in regulation of oxidative metabolism in adults of *Triturus viridescens*.

The metabolism of the tissues studied is summarized in Fig. 3. Oxygen uptake of liver slices obtained from 5°C acclimated frogs was higher than that from the 25°C group. The difference noted, however, was not significant even after 20 days of acclimation. The respiratory metabolism of skeletal muscle and stomach increased with time in the cold acclimated animals but decreased progressively in the group adjusting to the warmer temperature. The difference in oxygen consumption for these 2 tissues was highly significant ($p = .006$, muscle; $p = .002$, stomach) after 10 days. The general shape of the curves for these 2 tissues suggests that the changes in metabolic rate were the result of a gradual and cumulative process. Cardiac muscle (ventricle) responded in an opposite manner from the other tissues studied. The tissue from the 5°C group had a lower oxygen consumption than tissue obtained from the animals acclimated to 25°C. The significance of the differences ($p = .048$) noted was not as great as for the previous tissues but clearly indicates a change quite different from skeletal muscle. The metabolism of heart tissue reflected more closely the physical activity of the organ at the designated acclimation temperature.

In this study, oxygen consumption was expressed in terms of wet weight of tissue. Wet/dry weight ratios were determined at beginning and at end of the experimental acclimation period. From Table I, it will be noted that these wet/dry ratios were not different regardless of the experimental condition under which the animals were maintained. This finding upheld the validity of expressing oxygen consumption in reference to wet weight. In addition, these data rule out the possibility that the differences in metabolic rate noted in this study were due to changes of tissue water content.

Discussion. This study has demonstrated increased respiratory metabolism in frogs and Triturus acclimated to cold (5°C) as compared to animals kept at 25°C when the measurement was made at a common temperature. Fromm(8), in his study of seasonal variations of metabolism in frogs, reported decreases in oxygen consumption with refrigerated (4°C) frogs determined at 22-25°C. The reason for such diverse results is not clear, however, the present investigation agrees with the report

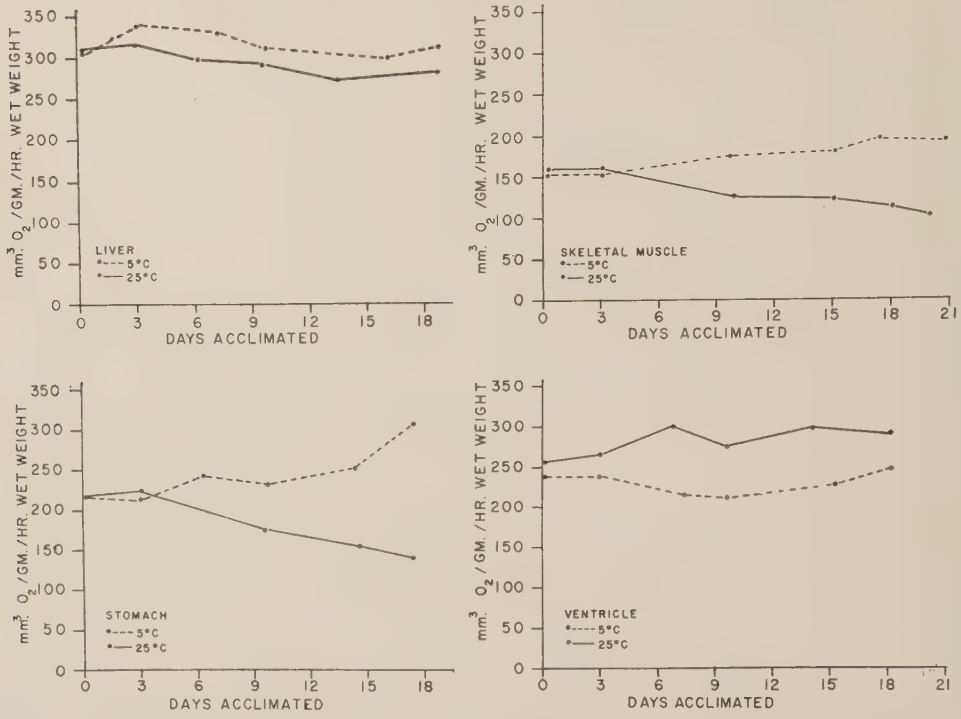

FIG. 3. Oxygen consumption of tissue slices from liver, skeletal muscle, cardiac muscle (ventricle) and stomach taken from frogs acclimated at 5°C and 25°C. Determinations of tissue slices were made at 25°C. Each point is mean from 8 determinations except for stomach at 18 days which is mean from 4 determinations and ventricle 25°C at 7 days which is mean from 5 measurements.

of Stangenberg(9) and her values for oxygen consumption agree closely with those in this experiment.

Tissue metabolism showed diverse changes with acclimation to temperature. It could reasonably be expected, that skeletal muscle, since it represents such a large portion of the body mass, would follow the pattern of changes exhibited by whole animal metabolism. This is, indeed, what was found in our study and is in agreement with Stangenberg (9). Goldfish skeletal muscle(1) demonstrates no change but this may be a species difference. It is interesting to note that ventricular muscle had changes of metabolism which would more closely parallel the physical activity at the acclimation temperature than the respiratory changes noted in the whole animal due to acclimatization. This unexpected finding cannot be clarified from the present data, but deserves fuller investigation. It may possibly be due to the phenomenon associated with measurements made on isolated tissues. It will be noted that all isolated tissues had a higher O_2 uptake per gram than did the whole animal at the same temperature.

The causal mechanisms in the changes of respiratory metabolism reported here cannot be clarified from data at hand. Changes in

TABLE I. Wet/Dry Weight Ratios and % Water in Tissues of *Rana pipiens*.

	Brain		Heart		Liver		Skeletal muscle	
	Wet/Dry	% H_2O	Wet/Dry	% H_2O	Wet/Dry	% H_2O	Wet/Dry	% H_2O
Normal*	5.85	(82.90)	5.88	(82.75)	4.11	(75.68)	5.45	(81.62)
5°C	5.88	(82.98)	5.80	(82.76)	4.16	(75.96)	5.47	(81.70)
25°C	5.80	(82.45)	5.70	(82.36)	3.89	(73.94)	5.30	(81.05)

* Normal tissue samples taken from animals kept at 20°C. Samples were taken at same time the groups were placed at acclimation temperatures. Each value is mean of 8 samples.

water content of the tissue were not a factor in the present investigation since no change was detected with acclimation. Stangenberg (9) reports a significant increase in samples of muscle tissue taken from animals kept at 6°C. These had a higher water content than those at 20°C. If water is a factor, does this mean that metabolism could be increased by artificially increasing water content of a given tissue?

Summary. Total body metabolism of *Rana pipiens* and *Triturus viridescens* was determined in animals acclimated to cold and warm environments. Those animals adjusted to cold had a higher oxygen uptake when measurements were made at an intermediate temperature. Thyroidectomy had no effect on respiratory metabolism of Triturus. Tissue metabolism of skeletal muscle and stomach had higher oxygen consumption when taken from cold acclimated (5°C) animals than when obtained from warm adjusted (25°C). Liver showed no significant changes. Ventricle responded just the opposite from skeletal muscle and stomach. The wet/dry weight ratios of all 4 tissues did not change during acclimation.

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Received December 12, 1959. P.S.E.B.M., 1960, v103.

CO_2 Retention in Anesthetized Dogs after Inhibition of Carbonic Anhydrase.* (25550)

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Berliner and Orloff(1) pointed out that an immediate effect of inhibiting the carbonic anhydrase of the blood "would be expected to be a diminution of the elimination of CO_2 and the accumulation of CO_2 in the body until a new steady state was reached." Such a steady state would require that elimination of CO_2 equaled its metabolic production. Measurements of CO_2 output 30 minutes or more after administration of carbonic anhydrase inhibitors revealed no such diminution(2,3). Mithoefer(4) made frequent measurements prior to 30 minutes, but found CO_2 output to be below the control measurement only in a dog in which ventilation was held constant. In the experiments reported here, particular attention was paid to the changes in CO_2 output occurring during the first 30 minutes after injection of a carbonic anhydrase inhibitor.

Methods. Dogs anesthetized with sodium pentobarbital were intubated with a tracheal cannula having an inflatable cuff so that an airtight seal was made within the trachea. Expired gas was collected in a Douglas bag from a respiratory valve attached to the cannula. Analyses of CO_2 and O_2 in duplicate samples of expired gas were made with a thermal conductivity type of CO_2 analyzer (Gow-Mac Gas Master) and a Beckman Model C oxygen analyzer. The volume of gas remaining in the Douglas bag was measured by a dry test meter, and corrected for the vol-

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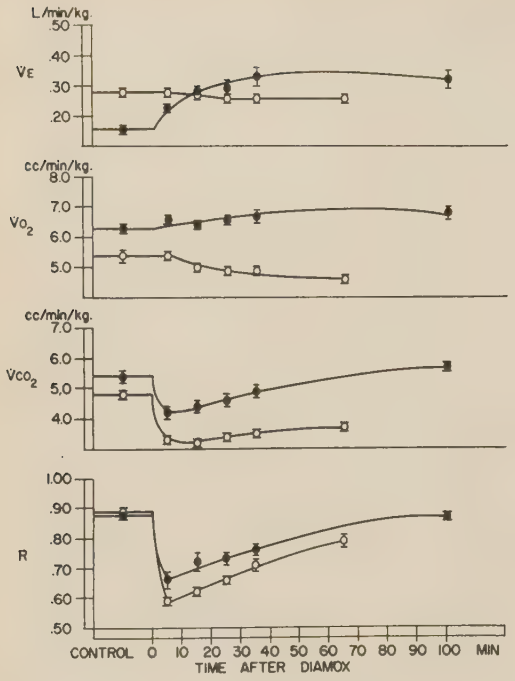

FIG. 1. Ventilation ($\dot{V}_E$), O_2 uptake ($\dot{V}O_2$), CO_2 output ($\dot{V}CO_2$), and respiratory exchange ratio (R) of dogs which ventilated spontaneously (●) and of dogs in which ventilation was controlled (○). Each point for the former is a mean of 6 animals and for the latter, 7 animals. Vertical bars represent ± 1 stand. error of mean.

umes taken for analysis. Expired minute volume of ventilation, O_2 uptake, CO_2 output, and respiratory exchange ratio were calculated from these data. Two series of experiments were conducted. The 6 dogs studied in Series A ventilated spontaneously. Collections of expired gas were made at least twice during the control period, at the end of which 50 mg/kg of acetazolamide‡ were injected intravenously. Five-minute collections of expired gas were completed at 5, 15, 25, 35, and 100 minutes after injection. In Series B, 7 dogs were used. Ventilation was kept constant with an intermittent positive pressure respirator supplied from a compressed air line. The dogs were not curarized, but were moderately hyperventilated in order to reduce voluntary respiratory efforts. Analyses and collections of expired gas were the same as for Series A dogs, except that the final collection was made 65 minutes after injection of actazolamide.

‡ The drug was injected as the sodium salt and contained 1.6 moles of NaOH for each mole of acetazolamide.

Results. Because there was little variation in the control measurements of individual dogs, and because collections of expired gas during the control periods of different dogs were not made at set intervals, all control measurements were pooled into a single average for each series (Fig. 1). After injection of acetazolamide, the ventilation of series A dogs increased within 5 minutes and was double the control value by 30 minutes. Because ventilation was artificially regulated in Series B dogs, there was little change from the control level.

O_2 uptake of Series A dogs tended to increase, but the greatest individual increase amounted to only 18% above the control. The dogs of Series B, on the other hand, showed a general trend toward a decrease in O_2 uptake after the drug. Much of the decrease was accounted for by one dog, in which O_2 uptake decreased 40% by the end of the experimental period. The other dogs in Series B showed much less change.

CO_2 elimination dropped sharply, on the average, in both series of dogs after injection of acetazolamide, although one individual in Series A showed no reduction. During the first 5 minutes, mean values for CO_2 elimination were 78% of control value in Series A and 69% of control value in Series B. Both of these changes were highly significant ($P < 0.01$). Following the initial drop, CO_2 elimination gradually rose. After about 60 minutes, the dogs of Series A reached control level of CO_2 output, but Series B dogs had not returned to control level by the end of the experimental period.

The respiratory exchange ratio decreased sharply in both series of animals after injection of acetazolamide, and then gradually returned toward normal. On the average, the dogs in which ventilation was held constant dropped to a lower ratio and were slower to return toward the control ratio. The indications were that both series would return to the control ratio, but during the experiment only Series A dogs actually reached it at 100 minutes.

Discussion. A decrease in CO_2 output when O_2 uptake is steady or rising and when ventilation is increasing can only mean an interference with the elimination of CO_2. The depression of CO_2 output in Series A dogs when ventilation was increased, and the greater depression in the dogs in which ventilation was controlled, show not only a retention of CO_2 relative to the ventilation, but also an absolute retention of metabolically produced CO_2. Mithoefer(4) reported similar experiments on one dog and found no reduction in CO_2 output, although alveolar ventilation increased 2-fold. Of the 6 dogs in Series A, only one was able to maintain CO_2 output at control level after acetazolamide. The other 5 dogs all had a decrease in CO_2 output in spite of increasing the total ventilation as much as 2-fold.

The CO_2 which was retained was the difference between rate of CO_2 production by the tissues and rate of CO_2 elimination at the lung. By assuming that metabolic production of CO_2 continued in the same proportion to O_2 uptake after acetazolamide as before, it was possible to estimate the volume of CO_2 retained. This was done by extending a line from control CO_2 output in Fig. 1 parallel to O_2 uptake. The area between the extrapolated line and the CO_2 output curve at any time was the volume of CO_2 retained at that time. For Series A mean volume of retained CO_2 at 60 minutes was 23 cc/kg, and for Series B, at 65 minutes, 79 cc/kg.

By assuming that the retained CO_2 would increase the CO_2 stores of the body just as any CO_2 loading, it was possible to estimate the rise in mean tissue Pco_2. Before this could be done, 2 corrections had to be applied to volume of CO_2 retained. The first was for amount of CO_2 bound by the NaOH injected with the acetazolamide. The second correction was to account for the CO_2 which would remain in the blood. The remainder of the retained CO_2 presumably was taken up by the tissues. When the volume of CO_2 retained in the tissues was divided by the slope of the dissociation curve for the tissues of the dog as determined by Farhi and Rahn(5), the rise in mean tissue Pco_2 was 10 mm Hg for Series A and 47 mm Hg for Series B.

Because the only difference in treatment of the 2 groups of dogs was maintaining ventilation constant in Series B, the increased ventilation of Series A evidently was an important factor in compensating for inhibition of carbonic anhydrase. If the gradient for Pco_2 from the tissues to the alveolar gas is considered, it is clear that it can be increased either by raising the Pco_2 of the tissues or by lowering the alveolar Pco_2. Series A dogs increased the gradient by both means. There was a greater rise in mean tissue Pco_2 of Series B dogs who could not lower alveolar Pco_2 by increasing ventilation. The conclusion reached is that when carbonic anhydrase is inhibited, an increase in the gradient for Pco_2 from the tissues to the lungs is essential for reestablishment of a steady state of CO_2 elimination.

Summary. CO_2 elimination of anesthetized dogs was temporarily reduced after injection of carbonic anhydrase inhibitor. Reduction was greater for dogs in which ventilation was held constant than for dogs ventilating spontaneously. Results indicate that increased gradient for Pco_2 from tissues to the alveolar gas is essential for reestablishing a steady state of CO_2 elimination when carbonic anhydrase is inhibited. The required increase in this gradient can be produced by means of augmented ventilation, by increased tissue Pco_2, or by combination of these 2 factors.

The authors wish to thank Richard F. Leeds and L. Paul Travis for technical assistance.

Acetazolamide (Diamox) was furnished by Lederle Labs., Am. Cyanamid Co.

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Received December 14, 1959. P.S.E.B.M., 1960, v103.

Isolation of Eastern Equine Encephalomyelitis Virus from *Aedes vexans* in Connecticut. (25551)

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Aedes vexans has been a mosquito suspected of transmission of eastern equine encephalomyelitis (EEE) since 1935, when Ten Broeck and Merrill(1) demonstrated in laboratory tests that this species was capable of transmitting the virus. It was further implicated in 1939, when Feemster and Getting(2) studied distribution of mosquitoes in Massachusetts following the 1938 epidemic and concluded that *A. vexans* was the most probable vector since it occurred in all areas where the disease appeared and was relatively more abundant than other *Aedes* mosquitoes during the fall season. However, EEE virus had never been isolated from collections of this species, and the purpose of this communication is to add *A. vexans* to the list of mosquitoes from which the virus has been obtained.

Methods. Epidemics of encephalitis among pheasants raised in Connecticut have occurred irregularly during the late summer and early fall for the last 2 decades. Each year since 1953, when EEE developed among penned pheasants at the Shade Swamp Fish and Game Wild Life Refuge in Farmington, Conn., this site has been used as a study area for investigation of the natural history of EEE. The study has included regular samplings of the mosquito population by taking biting collections, capturing adult mosquitoes in diurnal resting places, and examining larval breeding sites. In 1959, every week between March 1 and Nov. 1, adult female mosquitoes from these collections were transported in cardboard cartons to the laboratory where they were stupefied with cigarette smoke, sorted into pools by species (Table I), placed in small vials and stored frozen until processed in the virus laboratory.

The mosquito pools were triturated, either in a porcelain mortar with pestle or in a Ten Broeck grinder, then suspended in the proportion of one mosquito to 0.2 ml of Hanks' balanced salt solution. This solution contained antibiotics and was enriched by addition of 0.5% lactalbumin hydrolysate and 2% calf serum(3). The suspension was centrifuged at 10,000 rpm for 20 minutes and the supernatant inoculated into chick embryo tissue culture tubes as previously described(4).

Results. More than one-half of the 3194 mosquitoes collected were *Aedes cinereus* which, combined with *Aedes canadensis*, the next most numerous species, comprised 70% of the season's catch at this collecting site (Table I). Although *Aedes vexans* accounted for less than 5% of the specimens and only 10 of the 80 pools tested, the only virus isolation was made from a pool of 13 female *A. vexans*. The mosquitoes in this pool were hand-caught on Sept. 1, 1959, and contained no visible blood.

TABLE I. Mosquitoes Collected at Farmington, Conn., 1959, Number of Pools, Number of Specimens, and Number of Pools Positive for EEE Virus.

Species	No. pools	No. specimens	No. pools positive
Aedes cinereus	20	1675	0
canadensis	12	553	0
vexans	10	148	1
triseriatus	4	137	0
stimulans	1	18	0
grossbecki	1	3	0
Anopheles punctipennis	4	78	0
Culex pipiens	3	53	0
salinarius	2	39	0
territans	4	31	0
restuans	1	10	0
species	4	201	0
Culiseta morsitans	6	147	0
melanura	8	101	0
Totals	80	3194	1

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† This study was conducted under auspices of Commission on Viral Infections of Armed Forces Epidemiological Board and supported by Office of Surgeon General, Dept. of Army, and U.S.P.H.S.

On the third day following seeding of a suspension of this pool on tissue culture, definite cytopathogenic effects (CPE) suggesting the presence of a virus were noted. Subpassage produced CPE on the second day, and embryonated hen's eggs inoculated into the yolk sac were killed within 48 hours. The virus was identified as EEE by both hemagglutinating-inhibition and neutralization methods by titrating against a known EEE immune serum. The isolation was validated by reisolation of the virus from the original mosquito suspension.

Discussion. The sporadic incidence of EEE infections, lack of direct contact except among pheasants in the same pen, association of outbreaks with swampland areas and occurrence of infections following the peak of the mosquito season has long led to the suggestion that the virus was transmitted by mosquitoes. This theory has been supported by an accumulation of circumstantial evidence in recent years, despite the fact that virus has been isolated from several other arthropods. Howitt *et al.*(5) isolated it from mites (*Oermanyssus gallinae*) and chicken lice (*Eumenacanthus stramineus*) collected in Tennessee. The same workers isolated the virus from a mosquito (*Mansonia pertubans*) in Georgia(6). Chamberlain and co-workers(7) obtained the virus from the mosquito, *Culiseta melanura,* in Louisiana, and later from another mosquito, *Anopheles crucians,* in the same area(8). Holden *et al.*(9), in New Jersey, recovered virus from 3 pools of *C. melanura.* Recently, Karstad and co-workers(10) reported isolations from pools of *Anopheles crucians* and *Aedes mitchellae,* and from *Culicoides* species in Georgia. In 1956, during an encephalitis epidemic in pheasants in N. J., 4 isolations of EEE virus were obtained from pools of *C. melanura*(11), and during the same year 5 isolations were obtained from the same species in Massachusetts(12). This was strong evidence that *C. melanura* was a significant vector of transmission among birds. However, it was suggested in 1959 (13) that other species must still be considered, because in studies of the mosquito populations at farms in Connecticut where virus activity occurred between 1938 and 1956, including 20 farms at the time of pheasant and horse deaths, *C. melanura* could be found on only 3 of 25 farms. This species was considered rare in Connecticut, and the correlation of its distribution with that of the incidence of the disease could not be confirmed.

During these studies, however, *Aedes vexans* was found repeatedly, and although not always in great numbers, its distribution extended to all areas where virus activity occurred. This important epidemiological consideration, as well as the bionomics of the mosquito, led Feemster and Getting(2) to suggest it as a probable vector. They pointed out that *A. vexans* is a vicious biting mosquito. It has a wide variety in its host range and feeds readily on man, horses, cattle and birds. It commonly enters houses and barns, and the population peak occurs late in the summer just prior to the EEE epidemic season in the New England states. Now, with the isolation of the virus from *A. vexans,* this mosquito becomes more significant from the epidemiological standpoint.

It is interesting to note that the mosquitoes in the pool of *A. vexans* which yielded EEE virus were collected at Farmington, Conn., more than 3 miles from the nearest site where an epidemic of EEE occurred in penned pheasants during the current season. It is unlikely, therefore, that the mosquitoes had recently fed on infected domestic pheasants. It is more probable that they became infected by feeding on wild birds in the Shade Swamp Fish and Game Wildlife Refuge.

Summary. The virus of eastern equine encephalomyelitis was isolated from one pool of mosquitoes limited to 13 specimens of *Aedes vexans* collected Sept. 1, 1959, at Farmington, Conn. Distribution, seasonal incidence and bionomics of this mosquito fit epidemiological qualifications necessary for implication as a vector of eastern equine encephalomyelitis in this area.

The technical assistance of Jean Rawson and Paul Blodinger is gratefully acknowledged.

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Received December 15, 1959. P.S.E.B.M., 1960, v103.

Prevention by Stress and Cortisol of Gastric Ulcers Normally Produced by 48/80.* (25552)

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48/80, a product obtained by condensation of p-methoxyphenyl-methylamine with formaldehyde, is a particularly potent histamine liberator(1). Its most conspicuous toxic actions in the rat are anaphylactoid inflammation (with edema and cyanosis of the paws, snout, ears, and genitals) and acute hemorrhagic stomach ulcers with intense gastric edema(2). It is known that stress and glucocorticoids can also produce acute gastric ulcers, although they actually prevent anaphylactoid inflammation(3). The experiments reported here indicate that various stressors and cortisol inhibit all the just-mentioned toxic actions of 48/80, including the gastric lesions.

Methods. Eighty female Sprague-Dawley rats, with a mean initial body weight of 145 g (range: 140-154 g), were subdivided into 8 equal groups and treated as indicated in Table I. 48/80 was given intraperitoneally at the dose of 300 μg in 0.2 ml of water at 10 a.m. and 5 p.m. on one day only (Groups 1-7). The stressors employed were: Groups 3 and 8, *restraint* (animals strapped to board by adhesive tape in prone position 24 hours); Group 4, *cold* (rats were shaved and kept at 2°C for 3 hours in morning and 2 hours in evening); and Group 5, *motor denervation of extremities* (both cervical plexuses, as well as femoral and sciatic nerves, were severed under ether anesthesia). All these stressors were applied on day of 48/80 treatment, but one additional group (Group 2) was restrained on preceding day only, to show the effects of *pretreatment with stress.* In Group 6, *cortisol* was administered as suspension of microcrys-

TABLE I. Prevention by Stress and Cortisol of 48/80 Intoxication.

Group	Treatment	Shock + anaphylactoid inflammation	Gastric Ulcers	Gastric Edema
1	48/80	4+	4+	4+
2	48/80 + restraint (pretreatment)	0	Trace	Trace
3	48/80 + restraint	0	2+	0
4	48/80 + cold	0	Trace	0
5	48/80 + denervation of extremities	0	0	0
6	48/80 + cortisol (10 mg once)	0	Trace	0
7	48/80 + cortisol (1 mg/day, 10 days)	Trace	1+	Trace
8	Restraint	0	2+	0

* Supported by grant from Nat. Inst. of Arthr. and Metab. Dis., U.S.P.H.S., Consol. Grant Nat. Res. Council of Canada, and by Gustavus and Louise Pfeiffer Res. Found.

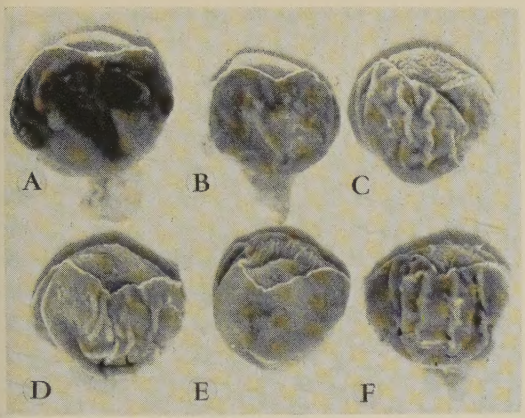

FIG. 1. Macroscopic appearance of gastric mucosa. A. 48/80; B. 48/80 + pretreatment with restraint; C. 48/80 + concurrent exposure to cold; D. 48/80 + concurrent denervation of extremities; E. 48/80 + pretreatment with single dose of cortisol; F. Restraint. Compare extensive hemorrhagic ulcerations and pronounced edema in A with virtually complete suppression of lesions in B, C, D, and E. In F, the ulcers are of the alarm-reaction type, unaccompanied by edema.

tals of its acetate, at dose of 10 mg in 0.2 ml of water, subcutaneously, once on day preceding 48/80 treatment, to produce intense but brief glucocorticoid overdosage. In Group 7, 1 mg/day of cortisol was administered in same manner during 10 preceding days, to establish the effect of a more chronic hormone pretreatment. All rats were killed with chloroform 24 hours after first 48/80 injection.

Results. In all control rats of Group 1, signs of shock and marked anaphylactoid inflammation, with edema and cyanosis of paws, snout, ears, and genitals, were seen after the first, and even more markedly after second, 48/80 injection, but none of these signs characteristic of histamine-liberator intoxication could be detected in the remaining groups, except for a trace of cyanosis and a slightly diminished spontaneous motor activity in rats of Group 7.

All control animals exhibited pronounced hemorrhagic gastric ulcers and intense edema of the gastric mucosa and submucosa. These changes were completely, or partially, prevented by various stressors (Groups 2-5) and by cortisol (Groups 6 and 7). Twenty-four hours of restraint (Group 8) is a particularly severe stressor, which elicits hemorrhagic gastric ulcers typical of the alarm reaction; these are essentially different from those induced by 48/80 (Group 1) for they are unaccompanied by gastric edema. Accordingly, when 48/80 and restraint were applied concurrently the gastric erosions of the alarm reaction developed, but were unaccompanied by edema. In rats restrained the day before treatment with 48/80, the erosions had disappeared by time of autopsy, yet the protection against the gastric lesion induced by 48/80 was still evident. Cortisol proved to be much more effective in inhibiting 48/80 intoxication when given as a single heavy dose on day preceding treatment with the histamine liberator than when the same total dose was injected during the 10 preceding days. These findings are summarized in Table I (which lists mean findings in terms of an arbitrary scale of 0-4+) and illustrated in Figs. 1 and 2.

Discussion. Gastric ulcers produced by the histamine liberator 48/80 differ qualitatively from those characteristic of the alarm reaction in that only the former are accompanied by marked gastric edema. Pretreatment with various stressors inhibits both production of anaphylactoid inflammation and

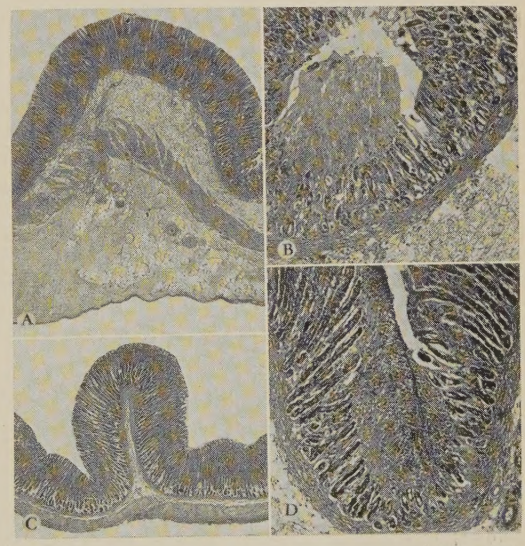

FIG. 2. Microscopic appearance of gastric mucosa. A and B, 48/80; C, 48/80 + pretreatment with restraint; D, restraint. Gigantic edema of submucosa and serosa (A) and necrosis with exulceration of mucosa (B) induced by 48/80 alone. Following 48/80 + restraint (C), gastric wall is normal; after restraint (D), necrosis with ulceration of mucosa but no edema.

induction of hemorrhagic edematous gastric lesions by 48/80. This effect of stress is presumably mediated through endogenous glucocorticoids, since it can be reproduced by pretreatment with cortisol. It had been noted that following pretreatment with 48/80 subsequent production of anaphylactoid inflammation by this same agent or other similarly acting substances is prevented. This had been ascribed to depletion of histamine reserve(4). Our observations do not invalidate this interpretation, but it must be kept in mind that the effect of such pretreatment may also be due to depletion of serotonin or of other metabolites necessary for development of an anaphylactoid inflammation and, indeed, may be the result of an alarm reaction produced by any stressor. The fact that histamine liberators greatly increase corticoid secretion(5) is consonant with the concept that even resistance induced by pretreatment with 48/80 and cognate compounds depends, at least in part, upon an increase in corticoid secretion.

Summary. Experiments on rats indicate that gastric ulcers produced by various stressors (as part of alarm reaction) differ qualitatively from those induced by histamine liberator 48/80. The latter lesions as well as the anaphylactoid inflammation and shock produced by 48/80 can actually be prevented by stressors or cortisol, although these same agents are, in themselves, capable of producing the alarm-reaction type of gastric erosion.

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Received December 18, 1959. P.S.E.B.M., 1960, v103.